POLYVITELLINY IN POND SNAILS.

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Introduction.

The occurrence of more than one vitellus, or embryo, in a single egg of some of the fresh-water pulmonates has never been satisfactorily explained. Although various forms of abnormal development which might be considered to have some relation to twinning, such as a *Campeloma* having two separate dextral shells (MacCurdy, '09), have been reported, such abnormalities were not observed in our laboratory stock or among the many wild individuals examined. The fact that a wild snail, number 5, Table I., was found to lay eggs having more than one vitellus when brought into the laboratory, suggested the idea that the laying of such abnormal eggs might be a transmissible character. When, however, in less than two months several of these abnormal, or polyvitelline, eggs had been laid by the controls and by other wild and laboratory reared snails, the genetic explanation seemed less likely. Fortunately, some of the stock from the projected

[1] Contribution from the Zoölogical Laboratory of the University of Pennsylvania and the Zoölogical Laboratory of the University of Michigan.

genetic experiment, the control and other isolated stock which was used in determining the viability of eggs laid by virgins (Crabb, '27*a*) furnish enough experimental data to show that the laying of polyvitelline eggs is not hereditary. Although the genetic experiment was discontinued, the occurrence of these abnormal eggs was noted in the records of other experiments.

Material and Methods.

Eggs of *Physa sayii, Lymnæa stagnalis appressa, L. columella* and *L. palustris* were used in this investigation. Egg masses of *L. s. appressa* and *L. palustris* containing polyvitelline eggs were placed in 5 per cent. formaldehyde for several hours, or even months; then the abnormal eggs were removed from the mass and mounted in hollowed slides, in 2.5 per cent. formaldehyde, to facilitate studying and drawing the vitelli. After a few weeks in the stronger solution the eggs ceased to shrink appreciably and began to darken but are still transparent after having been in the fluid eighteen months. The albumen of the eggs fixed in the strong solution over night and mounted in the weaker solution has neither coagulated nor blackened, and the vitelli are as plump and bright as they were when the eggs were sealed up over two years ago (Fgs. 3, 12).

In every instance the eggs recorded for individuals of each species represent all that that snail, or group of snails, laid during the period of observation. Thus, so far as the observations go, the data on the occurrence of polyvitelline eggs is much more reliable than if the masses had been collected at random.

Normal and Polyvitelline Eggs.

Normal eggs of the snails herein described consist essentially of an outer membrane enveloping a mass of albuminous substance which surrounds a relatively small, usually excentric, vitellus or yolk. Thus the structure of a pond snail's egg superficially resembles that of a bird.

Morphologically polyvitelline eggs of *L. s. appressa* and *L. palustris* differ from normal eggs only as regards the number of vitelli, and the amount of albumen displaced by the supernumerary vitelli. There is as much variation in size between normal eggs

(often of the same mass) as there is between normal and polyvitelline eggs. This is also true of the vitelli. Abnormal vitelli having the yolk protruding or flowing out, such as are shown in Figs. 3 and 11, and even cases of absence of vitelli, are occasionally found in eggs which to all other appearances are normal.

Several eggs have been found adhering together, but in most cases each one contained a single vitellus. In one instance (Fig. 7) two embryos are in a single egg while the other egg has none. In another case three eggs are joined together; the two end ones having no vitelli and the middle one having only two vitelli. The most remarkable instance of adhering eggs is one in which six normal eggs are joined in a string. However, there is nothing to indicate that polyvitelliny is dependent upon such adhesion of eggs.

The maturation and early cleavage stages are apparently normal in polyvitelline eggs having less than a dozen vitelli. In some eggs having a larger number many of the embryos pass the trochophore stage and some even reach the early shell stage before dying. The first maturation spindle is formed in a clear area or "well" in eggs which have been laid and moves to the periphery (Fig. 2) of the vitellus. The first polocyte migrates out into the albumen and the second remains attached, as has been shown is the case in normal eggs (Crabb, '27*a*).

We have never been able to hatch more than four snails from a single egg, but we have succeeded in repeating this experiment four times, and a fifth egg containing four embryos was killed and mounted. By tabulating the data available on the viability of polyvitelline eggs of *L. s. appressa* we find that of 53 eggs having two vitelli each, 39 hatched each vitellus, 8 hatched only one and 6 failed to hatch either vitellus. Of 5 eggs having three vitelli, 3 hatched all three, and 2 hatched only two of the vitelli. From 4 eggs containing four vitelli each, sixteen young were hatched. Of 116 *L. s. appressa* hatched from polyvitelline eggs not one was sinistral.

Occurrence of Polyvitelliny.

Twenty-one *Physa sayii,* fifteen of which were hatched from the same mass of eggs, deposited 348 masses containing 9,061 eggs, two of which had two vitelli each.

Of a large number of *Lymnæa columella* eggs taken at random only one contained more than one vitellus. This egg had two, one of which died in early cleavage and the other reached the gastrula stage.

Three F_1 and six F_2 of snail number 5, Table I., deposited a number of masses, which are not recorded in Table I., but none contained polyvitelline eggs.

Polyvitelline eggs occur most often in those masses which contain a large number of eggs (*i.e.*, 70 to 150 in *L. s. appressa*), and large masses are deposited by large, old individuals in the prime of life more often than small masses. This fact and the com-

Table I.

Normal and Polyvitelline Eggs Laid by Twenty *Lymnæa stagnalis appressa*.

Individual snails designated $1F_{11}$–$1F_{16}$ are F_1 progeny of snail No. 1. Likewise, those designated $4F_{11}$, $5F_{11}$ and $5F_{12}$ are the F_1 progeny of snails No. 4 and No. 5. Snails Nos. 1–4 and 11 were adults when collected from lakes near Ann Arbor, Michigan, while all the others were reared in the laboratory. All the F_1 individuals and those numbered 6–10 inclusive were reared in isolation. The masses for each individual are consecutive.

Individuals.	No. Masses.	No. Eggs.	Masses Containing Polyvitelline Eggs.	Polyvitelline Eggs.	Vitelli in Each Egg.
1	70	5,453	13	23	2(20 eggs), 3, 4, 4
$1F_{11}$	13	844	6	14	2(8), 3(4), 5, 32
$1F_{12}$	7	560	2	2	3, 3
$1F_{13}$	6	364	1	1	3
$1F_{14}$	6	491	1	3	2, 2, 3
$1F_{15}$	6	443	0	0	0
$1F_{16}$	5	441	1	1	2
2	50	2,506	5	7	2(6), 3
3	1	105	1	25	2(22), 3, 4, 4
4	1	64	1	2	2, 2
$4F_{11}$	22	1,608	8	25	2(22), 3, 4, 4
5	7	466	5	19	2(16), 3, 3, *ca.* 26
$5F_{11}$	20	1,631	14	131	2(39), 3(12), 4(11), 5(3), 7-9(3), 45, 6–15(29), 15–30(32).
$5F_{12}$	8	708	1	3	2, 2, 2
6	9	687	2	2	2, 2
7	8	497	0	0	0
8	4	246	0	0	0
9	20	935	0	0	0
10	7	487	0	0	0
11	14	1,327	6	15	2(15)
20	284	19,863	67	273	

Table II.

Normal and Polyvitelline Eggs Laid by about Eleven *Lymnæa palustris*.

The eggs in group 1 were laid by two snails kept in one aquarium; 2, eggs from an aquarium containing eight snails; 3, eggs laid by a single snail kept in isolation. The masses in each group are consecutive.

Group.	No. Masses.	No. Eggs.	Masses Containing Polyvitelline Eggs.	Polyvitelline Eggs.	Vitelli in Each Egg.
1.....	16	926	6	10	2(9), 3
2.....	91	4,824	1	1	2
3.....	21	1,189	2	4	2, 2, 3, 5
11.....	128	6,939	9	15	

paratively low number of eggs examined is probably the reason why polyvitelline eggs were not recorded for snails number 1 F_1 5, 7, 8 and 10, Table I. In the case of *L. palustris* the three individuals designated as groups 1 and 3, Table II., were large adults, but in group 2 only one was a large adult at the time we began examining the eggs. The data relative to the size and age of the twenty-one *Physa sayii* are not sufficient to determine whether or not the size of the individual and number of eggs in the mass are correlated with the presence or absence of polyvitelline eggs.

Discussion.

The facts set forth in this paper indicate that polyvitelline eggs may be expected to occur among the normal eggs of old *L. s. appressa* in the prime of life, but that the laying of these abnormal eggs probably is not a transmissible character. The question whether or not the two or more individuals hatched from the same egg are true twins, triplets, quadruplets, etc., has been raised. The paper of Hall ('25) appears to be the only available record of "twinning" in Mollusca. He found eggs of the tubiculous mollusc *Serpuloides vermicularia* containing two embryos which he considered twins. Newman ('23) holds that true twins arise from a single cell and that twinning is "essentially a phenomenon involving a physiological isolation of equivalent parts of the blastoderm and a regulation of the isolated or twinned regions into complete embryos." He also states that he has "never seen a reference to a case of twins or double monstrosity in Mollusca

. . . " and attributes this to the fact that cleavage is determinate in animals of this phylum. From his explanation and his review of the works of writers who claim that twins, at least in the forms they studied, are produced by a process of budding (Patterson), fission (Stockard) or fusion (Gemmill) it is evident that the process of twinning begins later than the first cleavage of the egg. However, it might be possible for two ova to fuse before either has undergone cleavage; whether this would produce twins or not is another question.

We have been unable to find anything to indicate that the supernumary individuals developed from pond snails eggs arose from a single vitellus. Although some four dozen ovotestes of *L. s. appressa* were sectioned (Crabb, '27*a*) not more than ten and seldom as many as four vitelli were found in the ovotestis and hermaphrodite duct in any one snail and in no instance were ova, and rarely sperms, found in the filliform part of the hermaphrodite duct (*i.e.,* craniad to the region " O," Crabb, '27*b,* Fig. 1) which in life bends around the gizzard. Thus it appears probable that while actively contracting the gizzard inhibits the passage of ova to such an extent that several ova accumulate in the enlarged part of the hermaphrodite duct and during a period of reduced activity of the gizzard all pass into the convoluted uterus at one time. In this, or some other way, a number of vitelli become enveloped by the albumen and the egg membrane which would normally cover a single vitellus. Often a mass of sperms is enclosed with the vitelli (Figs. 1, 5). Thus, probably by sheer accident, a polyvitelline egg is produced, and for this reason the young hatched from such an egg should not be considered true twins any more than should all the other young hatched from that mass of eggs.

Summary.

That polyvitelline eggs do not contain true twins is shown by the following facts:

1. The vitelli are normally enveloped by the albumen and egg membrane before any of them have undergone the first maturation division (Figs. 2, 6).

2. The vitelli often occur in uneven numbers (Plate 1, Tables I., II.).

3. Motile embryos show no evidence of attachment to each other.

4. Each of the 116 young was a normal dextral individual, except for size, when hatched.

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Plate I.

Figs. 1–6 and 10–13 are of *Lymnæa stagnalis appressa* eggs; Figs. 7–9 are of *L. palustris* eggs. All figures except Fig. 2 were drawn with the aid of the camera lucida to the scale shown in the plate. The eggs represented by Figs. 3 and 12 were mounted in a very weak solution of formaldehyde in a slide having the pits so shallow that the covers flattened them somewhat.

Explanation of Figures of Polyvitelline Eggs.

1. An egg having four vitelli. The dotted line represents the extent of a mass of sperms in the albumen.

2. Five unreduced vitelli occurring in a single section through an egg. The first maturation spindle is shown in one ovum, and an aster of a first maturation spindle is shown in another.

3. An egg laid by a wild snail, showing seven vitelli. Yolk globules have been extruded from four of the vitelli.

4. An egg having seven vitelli in sixteen to thirty-two-cell stages.

5. An egg with three vitelli in early cleavage stages. The dotted line indicates the extent of a mass of sperms in the albumen.

6. An egg having seven vitelli which have not given off the first polar bodies.

7. *Lymnæa palustris* egg with two advanced embryos, one of which probably belongs in the small attached egg.

8. An egg having two vitelli, one of which is in the four-cell stage.

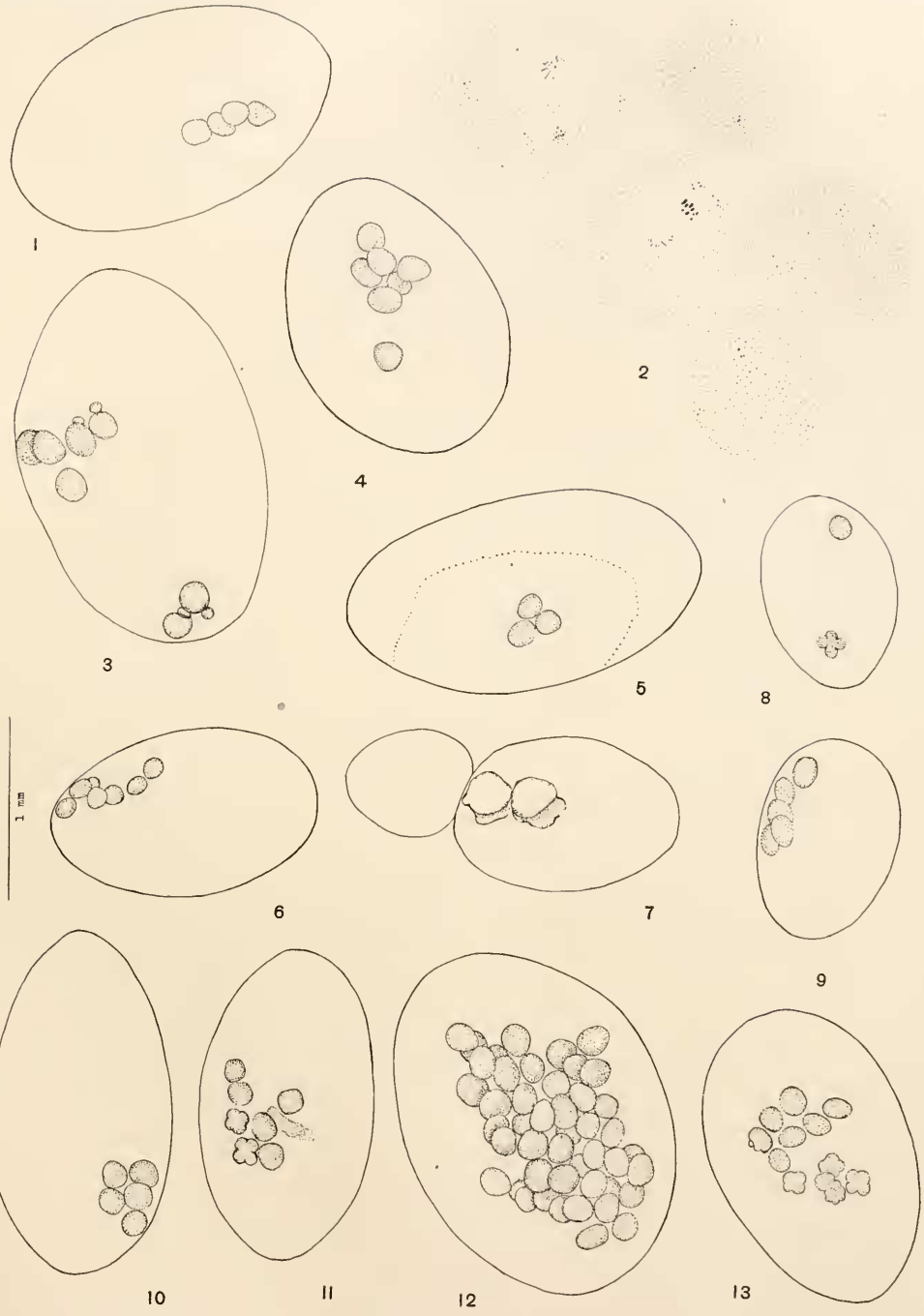
9. An egg having five vitelli in early cleavage stages.

10. *L. s. appressa* egg having five vitelli in early cleavage stages.

11. An egg having eight vitelli; one of which has disintegrated, while five are undergoing normal cleavage, and the other two apparently have not divided.

12. An egg having forty-six vitelli, laid by a wild snail.

13. An egg having twelve vitelli in four to eight-cell stages.



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THE EFFECT OF ALKALIES ON THE OXYGEN CONSUMPTION AND SUSCEPTIBILITY OF *PLANARIA DOROTOCEPHALA*.[1]

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I.

INTRODUCTION.

Considerable work has been done in this laboratory by Child and his students on the effect of chemical and physical agents on the modification of development and regeneration of organisms. For the interpretation of these results it is desirable that the action of the more commonly used chemicals on the rate of some metabolic process, such as respiration, be determined directly. Some work has already been accomplished along these lines, the effects of several substances on the rate of oxygen consumption having been tested: cyanides (Hyman, '19*a*), anesthetics (Buchanan, '22), caffein (Hinrichs, '24), and acids (Hyman, '25). The present paper is a contribution to this line of work and consists mainly of a study of the action of alkalies on the rate of oxygen consumption of *Planaria dorotocephala*. Hyman ('25) has already shown that acidification of natural water decreases the rate of oxygen consumption of *Planaria*. The question of the effect of increased alkalinity of natural water seemed of interest.

A number of investigations are available on the effect of increased alkalinity on various biological processes, such as activity, growth and development, and respiratory rate. Loeb ('98) found that the rate of embryonic development of *Arbacia* and *Fundulus* is accelerated in slightly alkaline solutions. Moore, Roaf, and Whitley ('05) also noted that bases in low concentrations favor growth and cell division in the fertilized eggs of *Echinus esculentus;* but Whitley ('05) failed to obtain any accelerating effect of

[1] Accepted as a thesis for the degree of Master of Science in the department of zoölogy of the University of Chicago.

alkaline solutions on the development of the teleost *Pleuronectes*. The fertilizability of *Arbacia* and *Asterias* eggs is increased by short exposures to alkaline sea-water according to Smith and Clowes ('24). Accelerations of activity by alkaline solutions have been observed by Dale ('13) in *Paramecium* and by Gray ('24) in the frontal cilia of *Mytilus edulis*.

Direct measurements of respiratory rate in alkaline solutions have yielded diverse results. In certain bacteria Brooks ('21, '22) noted the maximum rate of carbon dioxide production at or near neutrality with progressive decline in the rate with increasing alkalinity. The respiratory rate of the mold *Penicillium chrysogenum* is decreased 60 per cent. upon increasing the alkalinity of the medium from neutrality to pH 8.8 with NaOH (Gustafson, '20). According to Loeb and Wasteneys ('11) the rate of oxygen consumption of *Arbacia* eggs is increased 20–30 per cent. by raising the alkalinity of the sea-water with NaOH to pH 10.0 and increased 100 per cent. by raising it from pH 10.0 to 10.9. Similar results were obtained when ammonia was used. Thunberg ('09) found a decrease in the carbon dioxide production of excised frog muscle in solutions of NaOH, $Ca(OH)_2$, and $Mg(OH)_2$. Waldbott ('24) noted a slight acceleration of the respiratory metabolism of humans after the ingestion of alkaline solutions. The carbon dioxide production of tadpoles is increased in solutions of potassium and sodium hydroxide, but the rate of regeneration of the tail is retarded (Jewell, '20); Jewell regarded the increased respiratory metabolism as of a destructive nature.

From this review of the literature it is evident that both retardation and acceleration of biological processes result from exposure of organisms to alkaline solutions. In general, in the case of animal materials, an acceleration of growth and development and of respiratory metabolism in alkaline solutions has been found in the majority of cases.

Oxygen Consumption Experiments.

1. *Methods.*—The experiments consisted in determining the rate of oxygen consumption of planarians first in normal water

and then in water made alkaline to the desired degree by the addition of sodium or ammonium hydroxide. The general method of procedure was that described by Hyman ('19*a*). The species used was *Planaria dorotocephala*. Each experimental lot consisted of about 150 worms, 18–20 mm. long; these were selected from the laboratory stock and placed in a 500 cc. Erlenmeyer flask, in which they remained for a considerable time, being used in a number of experiments. They were fed in these flasks at intervals. At least three or four days were allowed to elapse after each feeding before the worms were used for experiment in order to avoid the increased respiratory rate consequent upon feeding (Hyman, '19*b*). The worms were fed sufficiently often to avoid any effects of starvation. From time to time new lots of worms were selected.

The water used was well water, an analysis of which is given by Hyman ('25). The alkaline water was prepared by adding enough sodium or ammonium hydroxide from stock solutions to raise this water to the desired alkalinity. The pH was determined with phenol red and thymol blue indicators by comparison with standard sets. At alkalinities greater than pH 8.6 a precipitate of calcium carbonate formed in the water. While there was no indication that this precipitate in any way affected the results, it was thought best to avoid it. This was done either by allowing the precipitate to settle and then decanting or by using carbonate-free water. This was prepared by adding 2 cc. concentrated HCl to eight liters of well-water and bubbling air through the water for 24 hours or more. That the rate of oxygen consumption of *Planaria dorotocephala* is the same in carbonate-free as in untreated well-water had previously been determined by Hyman ('25) and was verified in the present experiments.

Throughout each respiratory test, the flasks containing the worms and the blanks were kept in a large water bath at a temperature of 20° C. $\pm$ 0.5°. This was covered during the test. This darkening of the flasks together with the fact that the worms were kept continuously in the flasks was sufficient to eliminate movement. The worms remained very quiet throughout the oxygen consumption tests and the results cannot be ascribed to any differences in motor activity.

Two types of experiments were carried out: short exposures of a few hours to alkaline solutions, and long exposures of a week or more. Three experiments, that is, three flasks of worms, were generally carried on simultaneously.

2. *Short Time Experiments.*—In these experiments the rate of oxygen consumption of the worms was tested for two successive hours in normal well-water, pH 7.6 to 7.8. The water was then made alkaline by adding NaOH in varying amounts, giving a pH of 8.0 to 9.2 by intervals of 0.2 pH, or by adding NH_4OH similarly to alkalinities varying from pH 8.0 to 8.8. The rate of oxygen consumption was then tested in this alkaline water during the first third, and generally also fifth hours of exposure to it, freshly made alkaline water being used for each determination. As stated above, carbonate-free water was employed for alkalinities greater than pH 8.6. Twenty-three experiments were performed with sodium hydroxide and twelve with ammonium hydroxide. Table I. gives a typical experiment with each pH used for each of the two alkalies. The average per cent. of increase is based on all of the figures obtained in the alkaline solution. Table II. presents a summary of all of the short time experiments showing also the minimum, maximum, and average change in respiratory rate These tables show that the respiratory rate was sometimes decreased in alkaline solution but was more generally accelerated The normal variation in respiratory rate, based on twenty experiments where the respiration was determined in normal water for two successive hours, was 7 per cent. Only figures showing more than 7 per cent. alteration of the respiratory rate are therefore significant. From Table II. it can be seen that the higher alkalinities give generally a significant acceleration of the rate of oxygen consumption.

The worms would not survive alkalinities greater than those given in the table. They would live indefinitely in water made alkaline by NaOH to pH 9.0, but only twenty-four hours at pH 9.2. They were immediately killed in NH_4OH at pH 9.0. These results indicate that the action of alkali cannot be attributed solely to the hydroxyl ion.

It should be stated that the oxygen consumption of *Planaria* is independent of the oxygen content of the water at all ordinary

oxygen concentrations (between 7.0 and 2.0 cc. per liter, at least). The oxygen concentrations employed in the experiments was such (5 to 7 cc. per liter) that there is no possibility that the reduction in oxygen content by the worms during the experiment could have the slightest effect upon the amount of oxygen consumed.

Table I.

Results of a Typical Experiment, Short Time, at Each pH Used.

Figures represent cc. of oxygen consumed per hour.

	NaOH						NH_4OH			
Normal respiration in water. pH 7.6–7.8.										
	0.21	0.24	0.21	0.22	0.26	0.26	0.29	0.26	0.26	0.31
Respiration in alkaline water.										
pH	8.0	8.4	8.6	8.8	9.0	9.2	8.0	8.4	8.6	8.8
1st hr.	0.23	0.26	0.33	0.26	0.27	0.33	0.29	0.28	0.29	0.39
3d hr.	0.25	0.30	0.24	0.28	0.33	0.34	0.30	0.26	0.26	0.37
5th hr.	—	—	—	—	0.30	0.30	—	0.30	0.28	0.39
Average per cent. of increase of each of above.										
	14	16	32	22	15	20	1.5	7.6	6	23

Table II.

Average Percentages of Increase and Decrease Based on Total of All Short Experiments Done at Each pH.

Figures are ± per cent. from the normal.

	NaOH						NH_4OH			
pH	8.0	8.4	8.6	8.8	9.0	9.2	8.0	8.4	8.6	8.8
Total no. exp.	3	5	3	6	3	3	3	3	3	3
Increase and decrease in respiration rate from normal.										
Min.	−12	−12	+11	− 4	+ 3	− 7	−20	−18	−10	+19
Max.	+19	+28	+61	+86	+26	+58	+ 3	+15	+17	+32
Av. of all exp.	+ 6	+14	+32	+22	+17	+33	− 7	+ 3	+ 5	+25

The results of all of the short time experiments are given in graphic form in Fig. 1, made from the data given in Table II. The rate of oxygen consumption is given on the ordinate, normal respiration in untreated water being taken as 100, respiration in

alkaline water as per cent. decrease or increase from this. The alkalinity in terms of pH is given on the abscissa. The solid line represents the results with NaOH, the dashed line with NH_4OH. As already explained only differences greater or less than 7 per cent can be taken as significant. In the case of NaOH the experiments at pH 8.4 to 9.2 show on the average an increase above the normal variation, but there is apparently no relation between amount of acceleration and degree of alkalinity. The drop at pH 8.8 and 9.0 is probably not significant. In the case of ammonia the curve rises steadily with increasing alkalinity but probably only the increase at 8.8 is significant.

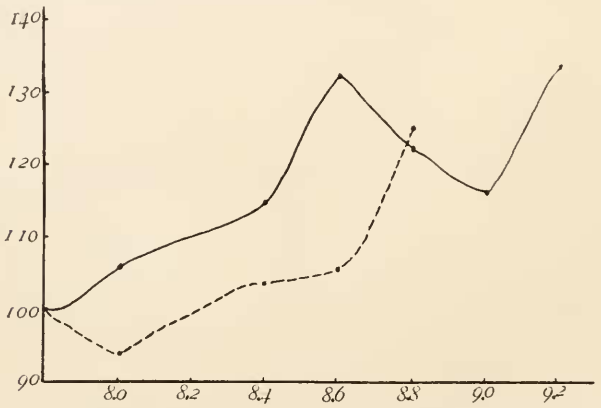

FIG. 1. Graph compiled from the average percentages of increase and decrease in respiration rate of all the short time experiments. See Table II. Per cent. of increase or decrease, normal respiration being taken as 100, on the ordinate and pH on the abscissa. Heavy line shows experiments in NaOH and broken line the experiments in NH_4OH.

3. *Long Time Experiments.*—These experiments consisted in determinations of the rate of oxygen consumption at frequent intervals in worms exposed continuously to alkaline water for periods of one to two weeks. Considerable difficulty was encountered in these experiments because the water would not remain at a definite alkalinity on account of the carbon dioxide given off by the animals. Various methods of keeping the water alkaline were suggested and were tried out. The carbonate-free water could not be used in the long time experiments, as, being unbuffered, it quickly became acid from the carbon dioxide given off by the

animals. Buffering this water with borax proved effective as a means of maintaining it at a definite alkalinity but the borax killed the worms in several days. Bubbling air through the flasks in the hope of removing the carbon dioxide emanating from the animals was not effective. When all of these methods failed, it became necessary to use the ordinary well water and to change it at frequent intervals. This proved fairly satisfactory. Boiling this water to remove dissolved carbon dioxide was of some help. The oxygen consumption of the worms was determined first in untreated well-water, pH 7.6 to 7.8. The water was then made alkaline to pH 9.0 with NaOH, decanted from the precipitate of $CaCO_3$ when necessary, and the worms kept in such water for one to two weeks continuously. The water was changed two or three times in 24 hours. Between changes, it became somewhat less alkaline but never fell below pH 8.6 throughout these long time experiments. The rate of oxygen consumption was determined every other day or every third day, always of course at pH 9.0. Owing to the fact that the worms lost weight during the experiment, because of starvation, for they were not fed throughout the period of experiment, it was necessary to weigh them in order to compare the rate of oxygen consumption at different intervals. The figures therefore represent the cc. of oxygen consumed per gram per hour. At the end of the experiment, the respiratory rate in normal water was again tested.

The results of three long time experiments which lasted one week are given in Table III., and of three more which lasted two weeks, in Table IV. In Table III., the oxygen consumption was determined first in normal water, then immediately in alkaline water (ph 9.0), then every other day in the alkaline water, and finally on the seventh day after the last test in alkaline water, again immediately in normal water. As shown in the table there was a marked acceleration of the respiratory rate during the latter part of the stay in alkaline water; this acceleration was immediately lost on return to untreated water, on the seventh day. The data in Table IV. give a similar result: acceleration during the exposure to alkaline water, immediate drop on return to untreated water.

Table III.

Table Showing Results of Long Time Experiments in Boiled Well Water, pH Raised to 9.0 with NaOH.

The pH never went below 8.6. Results expressed in cc. oxygen consumed per gram of animals per hour.

	1.	2.	3.	Av. % Increase 3 Exp.
I. day. Water pH 8.0	0.32	0.30	0.29	Normal. 100%
I. day. NaOH pH 9.0	0.28	0.28	0.30	− 5.5
III. day. NaOH pH 9.0	0.28	0.29	0.29	− 5.2
V. day. NaOH pH 9.0	0.44	0.26	0.41	+ 22.0
VII. day. NaOH pH 9.0	0.39	0.37	0.45	+ 33.0
VII. day. Water pH 8.0	0.24	0.26	0.25	− 17.0

Table IV.

Table Showing Results of Long Time Experiment in Ordinary Well Water Decanted off Precipitate.

The pH was raised to 9.0 with NaOH. The pH never went below 8.6. Results expressed in cc. oxygen consumed per gram of animals per hour.

	1.	2.	3.	Av. % Increase 3 Exp.
I. day. Water pH 8.0	0.21	0.18	0.19	Normal. 100%
I. day. NaOH pH 9.0	0.23	0.20	0.21	+ 10
III. day. NaOH pH 9.0	0.29	0.26	0.28	+ 43
VII. day. NaOH pH 9.0	0.24	0.23	0.24	+ 22
X. day. NaOH pH 9.0	0.24	0.23	0.21	+ 17
XIV. day. NaOH pH 9.0	0.27	0.29	0.23	+ 36
XIV. day. Water pH 8.0	0.23	0.20	0.18	+ 5

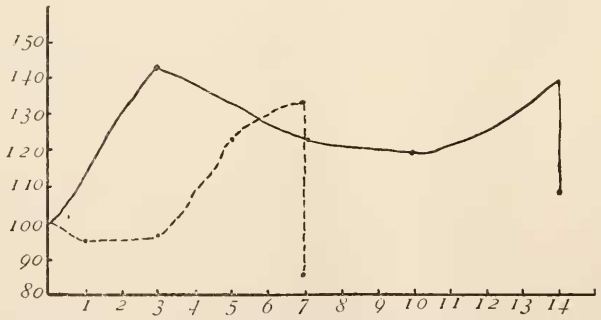

Fig. 2. Graph compiled from the data presented in Tables III. and IV. showing the results of long exposures of worms to NaOH solution, pH 9.0–8.6. Per cent. of increase or decrease, normal respiration being taken as 100, on the ordinate and number of days on the abscissa. Heavy line represents experiment in ordinary water and broken line experiment in boiled well water. The drop on the last day in each experiment shows the drop in respiration from NaOH solution to water.

The data given in Tables III. and IV. are graphed in Fig. 2, the solid line representing the data of Table IV., the dashed line, of Table III. The graph shows an acceleration of the rate of oxygen consumption during the greater part of the exposure to alkali, with an immediate drop at the end of each experiment on return to normal water.

Susceptibility Experiments.

The susceptibility method of determining differences in oxidative rate in different parts of the same organism and between different individuals of the same species was devised by Child and has been used extensively in this laboratory by him and his students. The method is explained by Child ('24). Briefly, the organisms to be compared are placed in toxic solutions of proper concentration or are exposed to lethal conditions and the various stages in disintegration are recorded. In general, the higher the oxidative rate, the shorter is the time before disintegration begins and the more rapidly does the disintegration progress. The method is therefore a rough means of comparing the relative rates of respiratory metabolism of comparable animals. Since the experiments on oxygen consumption had shown that the respiratory rate of *Planaria* is increased in alkaline solutions it seemed of interest to determine whether or not worms so accelerated would be more susceptible to chemical and physical agents than those respiring normally in ordinary water. A few experiments were tried to test out this point. Worms which had been kept for several hours in water made alkaline to pH 9.0 with NaOH were compared as to their susceptibility to toxic solutions and conditions while still in alkaline solution with control worms kept and tested in ordinary water or in solutions made up in ordinary water (pH about 8.0).

Comparison of susceptibility differences between different lots of worms is best accomplished by recording the progress of disintegration in certain arbitrarily selected stages. In the following experiments seven stages of disintegration were chosen: (1) worm entirely intact; (2) disintegration at the margins of the head; (3) from the end of marginal disintegration to complete disintegration of the head; (4) from the end of stage 3 to completion

of disintegration half way back to the mouth; (5) from the end of stage 4 to completion of disintegration to the level of the mouth; (6) from the end of stage 5 along the margins to the posterior end of the first zooid; (7) from the end of stage 6 to complete disintegration of the first zooid. The death of the secondary zooids is not considered. Ten animals are usually employed in each test, and the number of animals in each stage of disintegration is recorded hourly. The results are graphed by giving to each stage a numerical value, beginning with zero for stage 7, complete disintegration, and adding ten for each stage, intact animals being assigned the value of 60. The numerical value assigned to each stage is then multiplied by the number of animals in each stage at each observation. These values for the ten animals are then added together and divided by ten; the quotient thus gives the average stage of disintegration of the ten animals at the particular time of observation. Thus if at one observation, three animals were in stage 2, four in stage 3, and three in stage 4, their values would be 100,160, and 120, respectively, making a sum of 380; this divided by ten gives 38, or the average stage of disintegration reached, at the time in question.

1. *Experiments with Chemicals.*—To test the effect of alkalies on susceptibility to toxic chemical solutions, it was necessary of course to choose chemicals that were in themselves neutral. Anæsthetics were selected as the most convenient. Ethyl alcohol, 4 per cent., and chloretone, 0.1 per cent., were used. The anæsthetics for the control set of worms were made up in ordinary well water, those for the experimental set in the same water, made alkaline to pH 9.0 with NaOH. The results are shown in Figs. 3 and 4, being graphed according to the method explained above. Fig. 3 represents the results with alcohol, Fig. 4 with chloretone. In each graph, the solid line is the rate of disintegration of the control worms in ordinary water, the dashed line, the experimental worms in alkaline water. In each case, the disintegrating action of the anæsthetic is seen to be more rapid in alkaline solution.

2. *Experiments with Lack of Oxygen.*—When chemical agents are used the question of their penetrability is involved and complicates the interpretation of the results. For this reason it was thought advisable to kill the animals by other than chemical means

and determine the effect of alkalinity on the time of death. Lack of oxygen was one condition that seemed suitable for a test. A petri dish was divided into three compartments by two paraffin walls. A powder made by grinding up pyrogallic acid and sodium

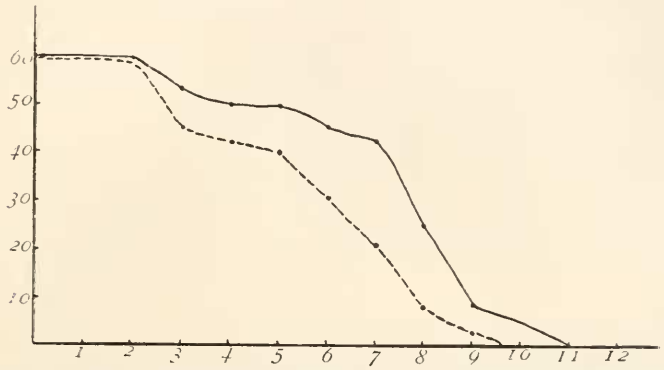

FIG. 3. Graph of disintegration gradient of worms exposed to NaOH solution and tested in alkaline alcohol (broken line) and worms not exposed to alkali and tested in neutral alcohol (heavy line). Alcohol used was 4 per cent. Hours on the abscissa and stages in disintegration on the ordinate. (See text for stages.)

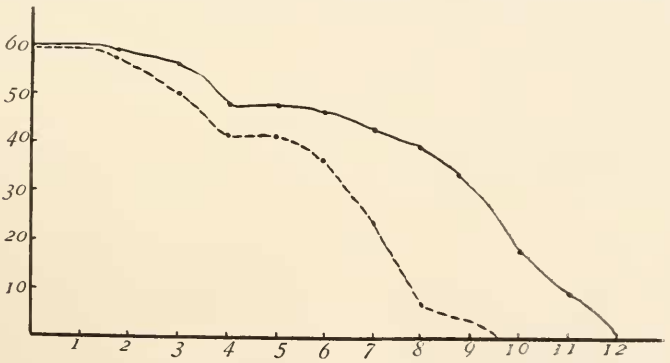

FIG. 4. Graph of disintegration gradient of worms exposed to NaOH solution and tested in alkaline chloretone (broken line) and worms not exposed to alkali and tested in neutral chloretone (heavy line). Chloretone used was 0.1 per cent. Hours on the abscissa and stages in disintegration on the ordinate. (See text for stages.)

hydroxide was placed in the center between the two paraffin walls. On one side of this were placed ten to twenty worms in ordinary water (pH 8.0); and on the opposite side in the third compartment, an equal number of worms of the same size and physiological

condition in alkaline water (pH 9.0). A cover was sealed on airtight. As the oxygen was absorbed by the alkaline pyrogallate, the worms began to die. The results of one such experiment are graphed in Fig. 5, the solid line representing the control worms,

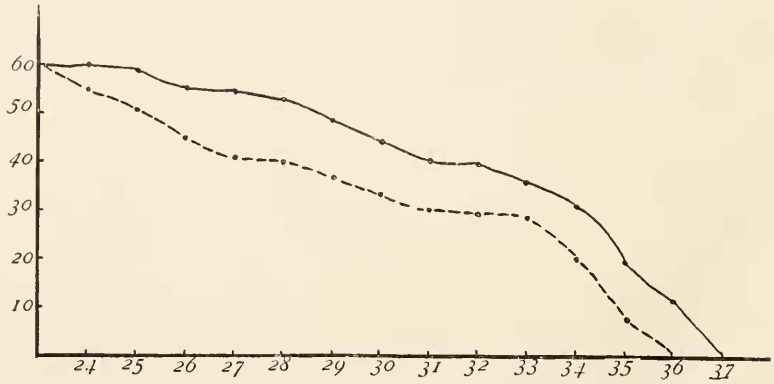

FIG. 5. Graph of disintegration gradient of worms exposed to NaOH solution and tested in alkaline solution in lack of oxygen experiment. Heavy line represents worms in ordinary water and broken line worms exposed to alkali. Record was not taken until disintegration began, *i.e.*, the twenty fourth hour. Hours are on the abscissa and stages of disintegration on the ordinate.

the dashed line, the experimental worms. In several experiments of this kind it was always found that the worms died faster from lack of oxygen at pH 9.0 than at pH 8.0.

3. *Experiments with Ultraviolet Radiation.*—Comparable lots of worms were exposed to ultraviolet radiation from a Cooper-Hewitt machine for a duration known to be lethal, one lot at pH 8.0, the other at pH 9.0. The worms were continually agitated during the exposure to insure uniform radiation. In all trials. the worms in the more alkaline water died more rapidly than those at the lower alkalinity. Death of course occurs only some hours after exposure to the radiation.

These experiments indicate that planarians are more susceptible to toxic chemicals and to lethal conditions when exposed to them in water of increased alkalinity, which is not in itself injurious.

4. *Susceptibility to Alkali.*—The question was raised whether the worms are able to acclimate to increased alkalinity. The long time experiments did not suggest that this was the case within two weeks' exposure, since the acceleration of the respiratory rate

endured through the period of the experiments. A further test by the susceptibility method was suggested. If any acclimation occurred, worms that had been kept in a non-injurious concentration of alkali should be less susceptible to a lethal concentration of alkali than control worms. Stocks of worms were kept in water made alkaline to pH 8.8 and 9.0, respectively, with NaOH, and were compared as to susceptibility to a higher concentration of NaOH with control worms living in ordinary water, pH 8.0. Tests were made every third day over a period of ten days. No difference in susceptibility between experimental and control worms was found. The resistance of *Planaria* to alkali was, therefore, not increased by continuous exposure, up to ten days, to alkaline water.

Discussion.

The experiments reported in this paper show that in general the rate of oxygen consumption is accelerated in water made more alkaline than normal, within physiological limits, by sodium or ammonium hydroxide. This increase in the respiratory rate lasts as long as the worms remain in the alkaline water, at least up to two weeks. Upon return to water of normal alkalinity, the respiratory rate drops at once. No evidence of any acclimation to the alkaline environment appeared in the course of the experiments. The oxygen consumption remained at a supernormal figure during continued exposure to the increased alkalinity. Susceptibility tests also showed no increased tolerance to alkali as a result of living for some time in water of increased alkalinity.

The question of the cause of the accelerated respiratory rate in alkaline water is of interest but the present experiments throw no light on the matter. It is rather generally accepted that alkalies increase permeability or have some other surface action (*e.g.*, Osterhaut, '14, and Warburg, '10). Such surface changes might well be the cause of the acceleration of the respiratory rate. It is not probable that penetration of the alkali into the interior is a factor in the acceleration for although ammonia penetrates cells readily it is believed that sodium hydroxide does not penetrate until the surface is actually injured. Since both alkalies caused an increase in the rate of oxygen consumption, the effect appears to be a surface one.

Not only is the oxygen consumption of *Planaria* increased with exposure to alkaline solutions but also the susceptibility to toxic agents and conditions is greater when such agents and conditions are applied in alkaline water. In the case of chemical agents, this increased susceptibility in alkaline solution might be ascribed to increased permeability. This explanation does not seem applicable, however, to the result with lack of oxygen and ultraviolet radiation. Exposure to these conditions is more rapidly lethal in water of increased alkalinity than in normal water. It seems necessary to conclude that the increased susceptibility of worms to chemicals and to toxic conditions when exposed in water of increased alkalinity is in some way associated with the accelerated metabolism of the animals in alkaline water. Susceptibility is thus again indicated as a method of measuring roughly differences in general metabolic rate.

Summary.

1. The general result of exposing *Planaria dorotocephala* to water whose alkalinity is increased from pH 7.6 or 7.8 to 8.0 to 9.2 by addition of NaOH or to 8.0 to 8.8 by addition of NH_4OH is an increase in the rate of oxygen consumption, whether the exposure is for long or short periods.

2. The increase lasts as long as the planarians remain in the alkaline water (longest experiment, two weeks). A return to the normal or to a lower rate (probably result of starvation) occurs at once when the animals are returned to water of the original pH.

3. The resistance of planarians to lethal concentrations of alkali is not altered by long exposure (ten days) to non-injurious concentrations of alkali.

4. The susceptibility of planarians to toxic chemical solutions, to lack of oxygen, and to ultraviolet radiation is greater when they are exposed to these conditions at pH 9.0 than when exposed at ordinary alkalinity of normal water (pH 7.8 to 8.0).

I desire to express my thanks to Professor C. M. Child and Dr. L. H. Hyman under whose direction the work was done.

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A CASE OF APPARENTLY ADAPTIVE ACCELERATION OF EMBRYONIC GROWTH-RATE IN BIRDS.

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Temperature experiments upon the egg of the domestic fowl have shown that the period of incubation varies inversely with the temperature at which the eggs are incubated. It may be assumed that this is due to an increase in the increment of embryonic development. If we consider any temperature well within the margins of safety for the egg (high 105° F. *ca.*, low 100° F. *ca.*) such as 102.5° F., we may say that an increase of one degree leads to an accelerated development while a decrease of one degree leads to a depressed or inhibited development. However, as far as I have been able to discover, no case is known in nature where an adaptive acceleration of the rate of embryonic development seems to have accompanied the evolution of new species within a group. The purpose of this paper is to report on what seems to be such an instance.

The cowbirds are a group of icterine birds comprising half a dozen species placed in three closely related genera:—*Agelaioides, Molothrus,* and *Tangavius.* Of these three, the first named is in every way the most primitive and doubtlessly represents the primitive stock from which the other two have evolved. The last mentioned is a side branch of the cowbird group and need not be considered in this paper. The species of *Molothrus* (and *Tangavius*) are parasitic in their breeding habits, *i.e.*, they do not make any nests of their own but deposit their eggs in nests of other birds and leave them to be incubated and hatched by these strange species. *Agelaioides* is not parasitic but is not entirely normal in its breeding habits. It breeds in old nests of other birds but cares for its own eggs and young. It will build for itself only if it cannot possibly get possession of a nest already built, showing that it still possesses some of its original nest-building instincts, but makes use of them only in case of emergency. In this genus the

female is extremely shy when on the nest and were it not for the protection of the male it is doubtful if in many cases the female would take sufficient care of the eggs. In *Agelaioides* the incubation period is 12½ to 13 days.

The genus *Molothrus* contains three species all of which are parasitic but have the parasitic habit developed to unequal degrees of perfection. The stages of perfection of the parasitic habit in these birds agree with what seems to be the phylogenetic relationships of the species. The most primitive member of the genus, *M. rufo-axillaris,* is parasitic on the still more primitive, non-parasitic *Agelaioides badius.* Its incubation period is the same as that of its victim and varies from 12½ to 13 days. The second species, *M. bonariensis,* is parasitic on a large number of small birds but has the parasitic habit very imperfectly developed, wasting large numbers of its eggs by laying in deserted nests, or even on the ground. The incubation period of this species is 11½ days. The third species of the genus, *M. ater,* has the parasitic habit best developed, lays its eggs in the nests of a great many species of birds, and does not waste its eggs as does *M. bonariensis.* This species has an incubation period of from 10 to 10½ days. (No bird in the world is known to have a shorter incubation period; few have one as short.) [1]

For the successful prosecution of a mode of reproduction such as parasitism implies it is obviously of great importance to the parasitic species to have a short incubation period as its egg may be laid in a nest after incubation has been started in the other eggs and yet must hatch as soon if not sooner than these others if the young parasite is to survive. (As a matter of fact in the majority of cases, irrespective of the species of bird victimized, the cowbirds' eggs hatch first.) Consequently when we find that with the perfection of the parasitic habit in the cowbirds (from the primitive non-parasitic *Agelaioides badius* to the relatively perfect *Molothrus ater*) there is a corresponding diminution of the period of incubation, or in other words, an acceleration of the embryonic growth-rate (amounting to about 20 per cent. difference between *A. badius* and *M. ater*) it is difficult to interpret the case as other

[1] The evidence on which the phylogenetic relationships of the species of cowbirds is based is contained in a manuscript now going the rounds of the publishers.

than adaptive. Shortness of incubation period could very easily have been of enough selective value for natural selection to operate on in a very decided fashion.

Lest it be thought that the differences in incubation period of the various species of cowbirds are due to differences in the extent of embryonic development, it should be noted that all of these birds hatch out in exactly the same stage of development. The size of the bird incubating the eggs seems to have no noticeable effect on the period of incubation. Birds as large as mocking-birds and as small as vireos hatch out young cowbirds in the same number of days. The size of the eggs of the various species of cowbirds is practically the same in all cases.

COMMENSAL ASSOCIATION OF A SPIDER CRAB AND A MEDUSA.

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During four successive years the writer was associated with a week-end biotal survey of the coast of South Carolina, and on two of these field studies encountered a curious commensal relationship between a spider crab and a medusa. The animals concerned were *Libinia dubia* Milne-Edwards and *Stomolophus meleagris* L. Agassiz, both of them common and typical inhabitants of the littoral zone of the Austroriparian Subregion.

Libinia dubia is a spider crab of moderate size with a rounded carapace averaging 6 cm. in diameter. It has long and slender walking legs and six median dorsal spines. Adult stages are confined entirely to the bottom zone where they crawl about in search of food. They are scavengers and members of the benthos.

Stomolophus meleagris is a rhizostome medusa of a diameter of 18 to 20 cm., hemispherical, without marginal tentacles, with eight rhopalia, and with fused oral lobes which form a thick cylinder, at the bottom of which are eight pairs of frilled lobes and a small central mouth opening. The margin of the umbrella is colored with a dark reddish-brown material, fading out aborally, which comes off freely and stains the hands when the specimens are picked up for inspection. They do not appear to possess any nettling organs, or at least none which could be employed defensively. This form is by far the most abundant Scyphozoan of the South Carolina coast, and is one of the more conspicuous planktonic organisms of the littoral zone.

Here we have to do then with a commensal relationship between a member of the benthos on the one hand and of vertically the most distant life zone, the plankton, on the other—certainly a most unusual arrangement. By means of this alliance the crab becomes a transient component of the plankton. The association

consists in the presence of the crustacean within the subumbrellar space of the medusa, clinging to the manubrium. Most of the crabs were observed with the head directed downward, but a few were situated with the head upward. In no case was more than one crab found in a single medusa. They resisted detachment, holding on tightly by means of the sharp and strongly curved ends of their legs, and would occasionally attempt to escape by scrambling around the manubrium, just as a squirrel does around a tree trunk. In such cases, however, their agility was not marked.

At Georgetown and at Murrell's Inlet, S. C., in the north-central region of the coastline, no examples of commensalism were observed. One *Libinia dubia* was taken on the rocks of the jetties at Georgetown, but no living medusæ were seen at either station. At Bluffton and at Sullivan's Island, in the south-central portion, the following records were obtained: Bluffton, three medusæ seined from relatively deep water, each containing a crab; no other examples of either species were seen. Between Sullivan's Island and the Isle of Palms, near Charleston, seventeen *Stomolophus* were taken by net and by hand from shallow water, and of these sixteen concealed a *Libinia*. In the remaining case, a crab was found on the bottom in the immediate vicinity of the jellyfish and the collector believed the crab to have just dropped from his conveyor, probably due to the commotion in the water which the numerous members of the party were causing. No other examples of either species were encountered, so this supposition is strongly probable. The commensal crabs varied in size but were all fully adult. At all four stations great numbers of stranded medusæ, mostly *Stomolophus* were seen along all beaches.

Thus it is seen that (1) along the South Carolina coastline both *Stomolophus* and *Libinia* are not uncommon and are frequently abundant; more so in the southern half, and especially true of the medusa as attested by the many cast-up specimens. (2) With a single exception, all examples of *Libinia dubia* captured were taken from the subumbrella of the medusa. (3) Again with a single exception, and that one a doubtful case, all examples of *Stomolophus meleagris* were found to harbor a crab. (4) All of the observations were made in the month of May and involved living adult stages of the commensals.

Several varieties of Hyperid Amphipods and also certain small fish are commonly known to seek shelter beneath the umbrella of various medusæ, but cases of large sized crabs occupying this situation seem to be rare. No references in the literature involving either of these types of animal as commensals were found by the writer, who is indebted to Dr. Mary J. Rathbun for the two following citations: Rathbun, '04, in writing of the distribution of Cancer jordani in Monterey Bay, California, notes "one under rocks between low tide mark and mean tide, and three from the subumbrella space of a large violet-and-white jellyfish" (species not stated). Weymouth, '10, writes: "*Cancer gracilis* is represented by a considerable number of specimens, both young and adult, all obtained by dredging, though in Puget Sound it is an abundant shore crab. On several occasions the young of this species has been found in considerable numbers clinging to the subumbrella of various medusæ. These have all been of small size—5 to 10 mm.—but it was not until the summer of 1908 that younger stages were found. On one occasion a number of medusæ were collected and in examining these many very young crabs were obtained and a smaller number of megalops. Some of these were kept alive until the molt to the young crab stage took place, so that there can be little doubt that the specimens were really the megalops of *gracilis*. Later megalops were obtained from other species of medusæ, and crabs of a slightly larger size than those found on the jellyfish were dredged in considerable numbers; it would appear, therefore, that in the case of *gracilis*, at least a considerable number of individuals pass that portion of their life history from the end of the free-swimming stage, probably early megalops, until reaching a size of 15 to 20 mm. clinging to medusæ, after which they drop to the bottom and live in the manner of the adult. I have found no other species than *gracilis* on medusæ though Miss Rathbun reports one specimen of *jordani* from the same situation. It would be interesting to know if this form of life history were universal with *gracilis* and if it were common in any other species." It will be noted that Weymouth is incorrect in the number of specimens observed as commensal by Dr. Rathbun, and also that no identifications of the medusæ involved are given.

The conclusion reached by Weymouth as to the cause underlying the association he reports is entirely plausible, but what is one to say in the case of *Stomolophus* and *Libinia?* It does not seem possible that either commensal was concerned with the food habits of the other; no crabs were observed feeding on dead specimens of the medusa, and though they may do so in deeper waters, this type of crustacean is not known to attack living prey. It is certain that the crab added the burden of freight to the jellyfish, but probably did it no other injury: also any possible benefit to the latter seems unlikely. On the other hand it is unquestionable that the crab received both shelter and transportation, though whether either or both of these benefits accounts for the condition is problematical. That the association has anything to do with the life history of the crab appears doubtful, since all specimens found were adults and long past the delicate stages of *Cancer gracilis* collected by Weymouth. The only other speculation which occurs to the writer is that the crab might resort to such shelter at the molting periods, but here again fact does not support theory, as those individuals observed all had fully hardened shells. The purpose of this curious union then remains to be determined by more extended and exact studies.

Another interesting angle of this situation is the problem of how the crab attains the medusa. Since the former is absolutely confined to the bottom in so far as its own efforts are concerned, there remain but two alternatives: either the medusa must descend to the substratum at least occasionally, and for an obscure purpose, or else one of the larval stages of the crab must seek shelter within the umbrella and then remain attached during a long period of its mature life, for a reason equally difficult to conjecture.

And if the facts of the case are not sufficiently strange, consider also the circumstance that this observation has so long escaped scientific detection and is also unknown to all of the local fishermen, boatmen, and sportsmen whom the writer interviewed, and this in spite of the familiarity of the animals concerned and the well nigh universality of the association in the region covered.

Spider crabs are famous for their protective adaptations, adorning the carapace with algæ, sponges, and hydroids, or possessing a shell so colored and sculptured as to blend effectively with the en-

vironment. Judging from this ever present condition, these crabs seem to require more than ordinary concealment from the numerous foes which prey upon them, and hence this factor may be the determining cause for their association with medusæ in the present case. In this connection it is of interest to point out a certain degree of mutual adaptation on the part of the associating species. *Stomolophus* has a spacious and deep subumbrellar area, within which its large guest finds snug but ample accommodation; the marginal aperture allows ready ingress and egress; the manubrium is deeply grooved and pitted, enabling the crab to easily cling to its steed with great tenacity; and nematocysts are absent or so poorly developed as to be ineffective. The *Libinia,* on its part, is admirably shaped so as to conform to the contours of this peculiar residence, the back being strongly convex and the abdominal surface slightly concave; the legs are curved inward and end in sharply pointed tips; and finally, the mid-dorsal spines of the carapace are well developed and would assist in maintaining the position, especially when the crab was situated in the upper regions of the subumbrella, the site from which all of the nineteen specimens here recorded were dislodged.

The writer wishes to acknowledge the assistance of Dr. Waldo L. Schmitt of the United States National Museum, who has kindly provided references and identification checks, by Dr. Bigelow for the medusa and Dr. Rathbun for the crab, at a time when original sources were not otherwise available. Specimens illustrating this association are deposited in the United States National Museum and in the zoölogical collections of the University of South Carolina.

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REGENERATION IN A TROPICAL EARTHWORM *PERIONYX EXCAVATUS* E. PERR.

G. E. GATES.

In the course of a recent study of the *Oligochæta* of Rangoon a number of earthworms were found with reproductive apertures in normal arrangement and position on the segment but displaced one to four metameres anterior to or one to three metameres posterior to their usual position. The prostates and ovaries had the same definite relations to the sexual orifices in such individuals as in normal specimens and hence were anterior or posterior to their ordinary position to an amount equivalent to the dislocation of the reproductive pores. None of the various metameric and organ anomalies that appear in nearly all of the species of Rangoon earthworms were noticed in specimens of the type just mentioned.

Morgan (1895) found similar anomalies in specimens of *Allolobophora fœtida* and suggested (p. 404) that "In those cases in which openings of the vasa deferentia occur on a segment anterior to the 15th metamere,[1] we may be dealing with a case of incomplete regeneration of the anterior metameres. . . ." But further on he says, "That all of the cases can be explained in this way is, I think, highly improbable," and (p. 450) "regeneration of the anterior end will not account for any of those cases where the vasa deferentia open on a matemere posterior to the fifteenth."

No references to hypermeric regeneration of anterior ends in earthworms could be found in the literature available. The abnormality (posterior dislocation) was found so frequently, however, that a series of operations was begun with the idea of learning whether or not such anomalous specimens would develop as the result of regenerative processes experimentally induced in earthworms of the species concerned. One of the animals first used seemed to have such an unusual capacity for replacing lost parts that an extensive series of experiments was initiated to discover the limits of this capacity. After several months it became

[1] In *A. fœtida* the normal position of the male pores is on segment fifteen.

necessary to discontinue the work. Some of the data accumulated are of such interest, however, as to warrant publication in a preliminary note. At the first opportunity the experiments will be resumed.

In the preliminary series of operations worms belonging to nearly all of the species occurring in Rangoon were anæsthetized and the anterior ends excised at various levels. Only individuals of two species, *Pontoscolex corethrurus,* and *Perionyx excavatus* survived more than five days after the operation. At the end of two months only a small amount of segmentally undifferentiated tissue had been produced at the cut ends of worms of the first species, whereas all operated specimens of the second species had regenerated, in much shorter time, segmentally differentiated anterior ends. Further experiments were confined to this second worm.

Adult specimens of *P. excavatus* attain a length of 130 mm., and a diameter of 5 mm. The prostomium is large, fleshy, and characteristic. The intersegmental furrows are deep and clearly marked. Secondary annulation is lacking. The dorsal and lateral parietes are heavily pigmented. The clitellum is ring-shaped, yellowish or gray, contrasting sharply in color with the non-clitellar segments, and lies between the intersegmental furrows 12/13 and 17/18. The setæ are numerous and arranged in a closed ring around each segment behind the first metamere. The spermathecal apertures are large paired pores in the intersegmental furrows 7/8 and 8/9 (paired spermathecæ in viii and ix). The single female pore is clearly visible on xiv (paired ovaries in xiii). The larger male apertures are closely approximated mid-ventrally on xviii (paired prostates in xviii, paired seminal vesicles in xi and xii, paired naked testes in x and xi).

Mature worms can be secured in large numbers all the year round. They easily adjust themselves to the conditions of life in the laboratory, and have an exceedingly low mortality rate after operation or injury. The sharply delimited clitellum and large sexual apertures enable rapid determination of position, while the clear cut intersegmental furrows, the absence of secondary annulation, and the distinctly projecting setal circles, render segment counting simple, and the detection of metameric and setal anoma-

lies easy. The dark red color of the normal segments sharply sets off the much lighter colored new tissue during the early weeks of regeneration and the presence of a protruding fleshy prostomium distinguishes a regenerating head from any other structure.

Only fully mature and normal worms were used. They were anæsthetized in a weak solution of chloretone and an excision was made by a clean cut, usually at an intersegmental furrow. The operated worms were kept in closed jars containing moist paper towelling. All results described in this paper were obtained in that part of the year known locally as the "cold season." The term is of course merely relative and indicates that the mercury is slightly lower all day long than at other seasons of the year. No effort was made to control the temperature but in the brick laboratory building the fluctuations of the mercury from midday to midnight are much less marked at this season of the year than out of doors.

In a short time after the operation a transparent conical outgrowth was visible at the cut end. In the case of regenerating anterior ends, oval fæcal pellets were found from the sixth day on, indicating that the digestive system had developed sufficiently in that short time to enable the worm to "bite" off, swallow, pass through the digestive system, and defecate pieces of the paper towelling. By the end of the second week the segmental differentiation of the regenerating heads was completed. Usually by the end of the third week the new segments contiguous to the old tail piece had attained the diameter of the metameres with which they were in contact, and were clearly setigerous. In the fourth week the pigment appeared in the regenerating segments.

Regeneration of Anterior Ends.

In all series of operations every surviving worm from which six or fewer segments had been amputated regenerated the exact number lost. If the prostomium or a fraction of the prostomium was removed a new prostomium or the missing fraction was replaced. In several worms a small wedge-shaped piece was removed dorsally, laterally, or ventrally from the anterior end of the stump. In each case such wedge-shaped pieces were replaced as well as the exact number of missing anterior segments. Only

one animal died following operation anterior to segment six and in this single instance there was reason for suspecting that the death of the worm was not caused by the effect of the operation on the individual but by another factor to be discussed later.

When the excision was made at an intersegmental furrow posterior to 6/7 but anterior to 18/19 the ratio of the number of segments removed to the number regenerated, varied and the percentage of failure to survive the operation was higher. Here again there was reason for suspecting that many of the mortalities following operation were due to something more than the effect of the operation itself. None of the amputations at intersegmental furrows 10/11 or 14/15 resulted in hypomeric regeneration, but the number of worms involved (five at the first furrow and two at the second level) is too small to permit generalization. At all other intersegmental furrows between 6/7 and 18/19 hypomeric anterior ends have been formed. The largest number of segments not replaced is four and the smallest is one.

In one series four worms regenerated hypomeric heads each having one "half segment," *i.e.*, a metamere extending across the whole diameter of the animal but antero-posteriorly only about half the width of the segments immediately in front of and behind it. Such half segments were not setigerous and may represent incompletely differentiated metameres, or perhaps growth zones, although the specimens concerned were killed in the fourth week after the operation, by which time the segmental differentiation is usually completed. In only one regenerating anterior end was a wedge-shaped half-segment found and this was ventral in position.

Excisions at levels 7/8, 9/10, 10/11, and 13/14 alone resulted in hypermeric regeneration but the total number of such cases is too small for generalization. Only one extra segment was formed in each case. In these hypermeric worms all the regenerated segments except of course the first were setigerous and clearly outlined by intersegmental furrows. No half-segments, wedge-shaped or otherwise were found in heads of this type.

When the anterior end was excised posterior to 17/18 only hypomeric heads of ten to sixteen segments were formed. The number of worms operated on behind this level was too small to

warrant a statement that total replacement of lost segments cannot take place posteriorly. It should be noted that when eighteen or more segments were amputated the digestive organs including part of the intestine which begins in the region of segment fifteen, as well as all of the reproductive organs were removed. When the cut was made between 17/18 and 24/25 twelve to sixteen segments usually regenerated. Posterior to 24/25 ten to fifteen segments regenerated. The posterior limit of anterior regeneration by tail pieces has not been determined, but it certainly lies unusually far back for earthworms, and at least in the last third of the length of the worm.

TABLE I.

SERIES TWO.

Anterior Ends from One to Twenty Segments Removed.

85 worms were amputated.
81 worms survived operation.
48 worms regenerated exact number of segments lost.
27 worms regenerated a smaller number.
5 worms regenerated a larger number.
1 worm failed to regenerate.

Table I. summarizes briefly the results obtained from a characteristic series of operations. Table II. summarizes the regeneration in the region lying between segments six and eighteen

TABLE II.

SERIES TWO.

Results of Operations between Intersegmental Furrows 5/6 and 17/18.

Number of Segments Removed.	Number of Specimens Operated.	Worms with Regeneration of Exact Number of Segments Lost.	Worms with Hypomeric Heads.	Worms with Hypermeric Heads.	Worms without Regeneration.
7	7	4	2	1	—
8	8	5	3	—	—
9	10	5	3	2	—
10	5	4	—	1	—
11	2	1	1	—	—
12	5	1	3	—	1
13	4	2	1	1	—
14	2	2	—	—	—
15	4	—	4	—	—
16	3	2	1	—	—
17	4	2	2	—	—

from the second series of operations. In Table III. is a condensed statement of hypomeric and hypermeric regeneration in the same region but including results of more than one series of operations.

Table III.

Hypomeric and Hypermeric Anterior Ends Regenerated at Cuts between Segments Six and Eighteen.

Number of Segments Removed.	Number of Segments Regenerated.
7	3, 6, 8
8	6, 7
9	6, 8, 10
10	11
11	8, 10
12	8, 10
13	11, 14
14	— —
15	11, 12, 13
16	14
17	13, 14, 15

Regeneration of Posterior Ends.

No special attempt was made to study tail regeneration by anterior pieces. Numbers of amputated anterior portions were kept in conditions similar to those of the regenerating tail pieces and the daily records of the experiments contain some notes on these amputated pieces. Anterior ends of more than twenty segments may regenerate new tails. No information is available as to tail regeneration anterior to segment twenty. Amputation at various levels posterior to 20/21 usually resulted in rapid formation of new posterior ends. None of these regenerating anterior portions were kept alive longer than four weeks so that it is not possible to make any positive statement as to the ratio of segments replaced to those lost. There is no reason to suspect, however, that anterior portions of twenty segments or more cannot replace all segments especially if provided with food.

Mutilations.

While the first series of experiments was under way a collection of worms containing several regenerating specimens of *P. excavatus* was brought into the laboratory. Arrangements were

at once made to secure several thousand worms of this species from various quarters of the town. A considerable number of individuals thus obtained had evidently been deprived in some manner of a head or tail or quite rarely of both ends. One collection of more than three hundred worms contained more than a hundred mutilated specimens. This percentage was so high that previous digging was suspected of being the cause of the mutilations. As the collections had been made on successive days over a period of several weeks, it is possible that some at least of the mutilated specimens were produced in this way. In order to avoid this factor, collections were made at several localities which presented every appearance of having been undisturbed for months. Such collections also contained high percentages of mutilated individuals. Practically all the mutilations found were amputations of a head or a tail at an intersegmental furrow. Only three regenerating individuals were found in which excision had occurred in the middle of a segment. In these specimens the missing half segment had been regenerated as well as a portion of the tail behind. One worm mentioned elsewhere had been deprived of dorsal portions of two segments in addition to the anterior end.

Table IV. summarizes the information available from records of the collections. It should be noted that all mutilations included within this table had been produced at least several days previous to the time of collection. Such few specimens as were brought into the laboratory obviously injured as the result of collecting processes were, of course, discarded and not included in the tables.

Through such collections more than fifteen worms were secured that had lost their heads. Nine were regenerating new anterior ends when brought into the laboratory. Of this latter number four were either immature or if mature had lost more than eighteen segments, for there were no characteristic sexual markings to make a determination possible. The remaining five specimens had lost their heads anterior to the prostatic segment. By assuming that the prostatic segment of each of these animals was the eighteenth metamere before the mutilation, as in normal worms, it was possible to determine the number of segments lost and the type of regeneration that ensued. On the basis of this assump-

tion three individuals had regenerated hypomeric anterior ends, one had produced a hypermeric anterior end and the others had exactly replaced the number of segments lost. The single worm with hypermeric regeneration had lost the first thirteen segments as well as dorsal portions of segments xiv and xv and had not only replaced the lost dorsal portions but had also formed *fifteen* perfectly normal and clearly outlined setigerous segments and the non-setigerous prostomial segment.

Table IV.

Collection Number 1.

Normal worms	231
Mutilated worms	104
Worms with tail mutilation	98
Worms with head mutilation	6
Worms regenerating a tail	77
Worms regenerating a head	3

Collection Number 2.

Normal worms	301
Mutilated worms	87
Worms with tail mutilation	84
Worms with head mutilation	3
Worms regenerating a tail	71
Worms regenerating a head	2

Collection Number 3.

Normal worms	1
Mutilated worms	6
Worms with tail mutilation	5
Worms with head mutilation	1
Worms regenerating a tail	3
Worms regenerating a head	1

Collection Number 4.

Normal worms	?
Mutilated worms	?
Worms regenerating a tail	49
Worms regenerating a head	4

It is evident therefore that both in its natural environment and under experimental conditions in the laboratory *P. excavatus* may regenerate hypermeric anterior ends. In view of this demonstration there seems to be no need for further search, at least for the present, for other explanation of the origin of the anomalous

specimens mentioned at the outset of this paper. Such abnormalities at least in the species under discussion appear to be adequately accounted for as the products of hypo- or hyper-meric regeneration.

Varia.

Additional extensive experiments were begun to obtain information as to the posterior limit of anterior regeneration by tail portions, the anterior limit of posterior regeneration by head portions, the regeneration of pieces with two-cut ends from various regions of the body, etc. These experiments failed completely, as far as the objects in view were concerned, because of a constant series of accidents which will be described under the title of autotomy. A few notes from the records of these experiments are given herewith to indicate more clearly the unusual regenerative characteristics of this worm.

A. A short piece of eight segments from the middle portion of worm 109 was still living on the eighth day after the operation and responded quickly to various sorts of stimuli. Both ends had healed over without any signs of regeneration.

B. The forty-one anterior segments of worm 110 regenerated in nine days new tissue five and one half millimeters in length.[1] and fixing.

The new tail was composed of a long, metamerically differentiated portion containing more than thirty segments, the anal segment, and between these two a short region of formation of new metameres.

C. The anterior end of worm 132, a piece fifteen millimeters in length, composed of twenty-four segments regenerated in two weeks a tail fifteen millimeters long containing in the segmentally differentiated region, fifty-four segments.

D. A shorter anterior end from another worm regenerated at its posterior cut surface a *head* of several segments with a characteristic mouth and prostomium.

E. A twenty-three metamere fragment thirteen millimeters in length from the posterior half of worm 118 in two weeks regen-

[1] The measurements noted were made on material that had been killed by dropping into strong methylated spirits and then hardened in formalin. Old and new tissue appeared to be uniformly contracted by this mode of killing

erated at one end a new head containing thirteen segments and a prostomium and at the other end a tail two and one-half millimeters long, containing in addition to the anal segment and the growing region twelve differentiated segments.

F. A shorter portion from the posterior half of another worm regenerated at one end a head and at the other end a single anal segment.

G. A short tail fragment regenerated at its injured anterior end a structure exactly similar in appearance to the tail developed at the cut posterior surface of an anterior piece.

H. The nine anterior segments containing both pairs of spermathecæ were removed from worm 82. Eight segments regenerated. Characteristic spermathecal pores appeared in intersegmental furrows 6/7 and 8/9 (the posterior pair of pores being located between the last of the old and the first of the new segments!).

I. Several other worms from which anterior ends containing one or both pairs of spermathecæ had been removed regenerated heads with one or two pairs of spermathecal pores at various intersegmental levels. These worms were killed three weeks after the operation, hardened in formalin and dissected. Definitive spermathecæ had not been formed by that time. The site of each spermathecal pore was marked internally by the presence of a lump of soft spongy tissue. Some of these specimens with regenerated spermathecal pores were very similar to specimens of this species secured by Beddard (1886) from the Philippines. It is quite possible if not probable that many or even all of the thirteen anomalies described and figured by Beddard as "variations" were the result of regenerative processes.

J. Seventeen anterior segments were removed from worm 149. When the animal was killed at the end of the fourth week after the operation, seventeen segments had been regenerated. In the usual position on segment fourteen was a typical female pore. The clitellar segments (xiii–xvii) were distinctly lighter in color than the other new segments, indicating the beginning of clitellar differentiation. Although the head was carefully fixed and hardened the tissues were too soft and spongy internally for dissection and no reproductive organs could be demonstrated.

Autotomy.

Several references have already been made to disturbing factors which interfered with the success of some of the experiments. One of factors, the most important, was a tendency for the worms to break into fragments in early hours after the operation. For want of a better term this process of fragmentation will be referred to as autotomy. Only very rarely was this fragmentation observed to occur later than the first twenty four hours after the operation, and then only very small portions usually consisting of one or two segments were thrown off.

In the first series of operations on *P. excavatus* many of the operated worms autotomized one or more pieces from the posterior portion. In another series of anterior operations thirty two out of forty two animals autotomized portions of the tail ranging from seven to sixty millimeters in length. No series of operations in which tail portions of the worm were watched was free from this tendency to fragment. In the majority of cases one or two short pieces were autotomized from the posterior end of the major operated portion. Such fragments were usually dead when discovered but very often lacked the pungent odor so characteristic of decaying earthworm. Occasionally the fragmentation was much more striking and extensive. Worm *B* 9 from which eleven anterior segments had been removed broke into pieces. Worm *D* 19 from which *X* anterior segments had been removed broke entirely into pieces six to ten millimeters in length. Several other specimens from which eight or nine segments had been removed also autotomized extensively.

Autotomy is usually understood to be a throwing off by the animal of a small portion which usually dies without producing a new animal but in *P. excavatus* apparently any fragment from any region may survive, or more than one of the fragments may survive, with the survival determined by the presence or absence of something in the worm and not by the position of the fragment along the axis of the animal. The autotomy was observed only in posterior portions. The length of the tail however was of no significance. Posterior portions from one third to approximately nine-tenths of the length of the original worm autotomized

extensively while anterior portions longer than one-half never autotomized. Several posterior portions about equal to one-half the length of the original worm autotomized from each end one or two pieces which died while the longer middle portion survived.

The experiments were discontinued before a thorough analysis of various aspects of this interesting tendency to fragment could be completed. Two experiments, however, provided a hint, not only as to the cause of the fragmentation but also as to the cause of certain other disturbing factors. Worm 94 was anæsthetized as usual and cut into three approximately equal portions, each of which was kept separately in a tightly closed jar. No autotomy occurred in any of the jars. The head piece survived and regenerated a tail. The middle piece regenerated at one end an anal segment and at the other end a head about three millimeters in length composed of fifteen segments. On the day following the operation the tail portion was collapsed and flattened, dead, but without noticeable odor of decay. In the jar were three flies which must have been present in the tail portion of the worm at the time of the operation, as the jar was not opened until after the flies had appeared. Through the kindness of entomologists at the Imperial Bureau of Entomology, London, these flies have been identified as *Aphiochæta scalaris* Lw.

A number of head portions ranging from twenty-five segments to about half the length of the worm were kept in a single, large, tightly-closed jar. A few portions died during the first three days after the operations and were removed. At the end of the week four of the head pieces were still living and apparently in good health although without signs of regeneration. The only traces to be found of the other head portions were numerous tubular fragments of transparent cuticle. Crawling around inside the jar were numerous small insect larvæ. When the jar was opened two small flies very similar in appearance to those from the other worm escaped. There seems to be no reason for doubting that some larval stage of the fly was parasitic in these worms at the time of the operation and it is at least possible that the presence of parasitic fly larvæ is the factor responsible for the autotomy as well as other disturbances in the operated worms. It should be noted, however, that *A. scalaris* is a very general feeder and that

it has been bred from all sorts of decaying matter. The Director of the Imperial Bureau of Entomology writes: "I should be inclined to suppose that the attack on the earthworms that you have noticed was accidental, for it seems unlikely that this fly would prove to be a true parasite."

Summary.

1. *P. excavatus,* an earthworm occurring in large numbers in dung heaps and soil rich in decomposing organic matter in Rangoon has a regenerative capacity very much higher than any known at present from megadrilous Oligochæta with the single exception of the limnic *Criodrilus lacuum* Hoffm., from Europe. The rate at which regeneration is completed is rapid.

2. Posterior portions can replace the anterior segments lost if the number of metameres removed is seventeen or less. When more than seventeen segments are removed only ten to fifteen metameres were regenerated.

3. The posterior limit of head regeneration lies somewhere in the last third of the length of the worm.

4. Spermathecal apertures and female reproductive pores may develop on regenerating anterior ends.

5. Anterior pieces of twenty segments or more may regenerate tails.

6. A heteromorphic head may be regenerated at the posterior end of a very short anterior piece.

7. A heteromorphic tail may be regenerated at the anterior end of a very short tail piece.

8. A piece of twenty or more segments from the middle of the worm may regenerate at one end a tail and at the other end a head.

9. Regenerated heads may be normal, hypomeric, or hypermeric. Hypomeric and hypermeric regeneration is considered an adequate explanation of the origin of abnormalities described as anterior or posterior dislocation of the reproductive organs.

10. In collections made in various quarters of the town a high percentage of the individuals secured had been mutilated by the amputation of a head, a tail, or both. Many of the mutilated specimens were regenerating the lost parts when collected.

11. One or more pieces both anteriorly and posteriorly are very frequently autotomized after operation by posterior portions. Sometimes the whole tail portion fragments into pieces six to ten millimeters in length. Anterior ends have not been observed to autotomize.

12. A fly *A. scalaris* Lw. has been bred from portions of *P. excavatus*. Parasitism by this insect may possibly be responsible for the mutilated specimens and for the autotomy following operation.

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STUDIES ON THE PHYSIOLOGICAL EFFECTS OF HYDROGEN CYANIDE.

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The question of the physiological effects of cyanide has been of considerable interest since Claude Bernard (1) in 1857 noticed that the venous blood of vertebrates is bright red after treatment with cyanide. It is well known that cyanides in dilute solutions act in general as protoplasmic depressants. In most cases this depressing effect can be attributed to the inhibition of oxidations. Investigations by Allen (2), Child (3), Hyman (4, 4*a*, 4*b*), Vernon (5), Buchanan (6) and others show that potassium cyanide, even in extremely dilute solutions, depresses reversibly the rate of respiration in *Planaria*. Lund (7), however, noted no decrease in oxygen consumption in *Paramecia* placed in potassium cyanide solutions. The fact that dilute solutions of cyanide act as anesthetics is equally well known (Heilbrunn, 8; Osterhout, 9). An important difference between the effects of a typical anesthetic, such as ether, and cyanide was pointed out by Heilbrunn (8) who showed that ether decreases the viscosity of protoplasm while KCN in anesthetic concentrations increases it. Heilbrunn, therefore, concluded that in the case of sea urchin eggs there are two types of anesthesia; in one the viscosity of the cytoplasm is decreased and in the other it is increased. The toxic action of cyanide in concentrated solutions is also well established (Hyman, 4). In vertebrates the toxicity of cyanide seems to be due to its effect on the central nervous system, as shown by Geppert (10) and Dontas (11). Child (12) showed that the portion of an organism with the highest rate of metabolism is most susceptible to cyanide, and that young organisms having a high rate of metabolism are more susceptible than adults with lower rates. For a detailed review of the literature on the various phases of the cyanide problem, the reader is referred to the paper of Hyman (4).

The question of the effect of cyanide on the permeability of membranes is a debatable one. It is generally considered that anesthetics, such as alcohol and ether, decrease permeability (Lillie, 13; Lullies, 14; McClenden, 15; Osterhout 9*a*). Wertheimer (16), on the other hand, concluded that narcotics increase the permeability of frog skin while Krehan (17) showed that KCN increases the permeability of plant cells to many substances.

Most workers with cyanide have used potassium cyanide, which in an aqueous solution is strongly alkaline, due to the manner in which it dissociates:

$$\begin{array}{c} KCN + H_2O \rightleftharpoons KOH + HCN \\ \Updownarrow \\ K + OH \end{array}$$

Hydrogen cyanide in an aqueous solution acts as an extremely weak acid, dissociating only to a slight degree. In view of the fact that the question of the effect of cyanide on permeability is a debatable one and since most of the previous workers have used KCN, it was thought advisable to study in detail the penetration of hydrogen cyanide through living membranes as well as its effect on the membrane.

The investigations were conducted at the Zoölogical Laboratory, University of Pennsylvania, for which privilege the writer wishes to acknowledge his indebtedness to Doctor C. E. McClung. The writer is also under obligations to Doctor J. H. Bodine, under whose direction the investigations were conducted, for many helpful suggestions throughout the progress of the work.

It was found convenient in this work to use the artificial "cell" devised by Jacobs (18) and constructed in the following manner: a hard glass tube 7 cm. long and 1.5 cm. in diameter was tapered at one end to an opening of one cm. in diameter and the tapered end provided with a tip. The skin from the hind legs or back of a freshly killed frog (*Rana catesbiana* or *R. pipiens*) was carefully stretched over the lipped end of the tube and held in place by a rubber band. The skin was so placed over the tube that the inside, or flesh side, of the skin was exposed to the exterior. The "cell" so constructed was placed in a 100 cc. quinine bottle and

both the " cell " and bottle fitted with rubber stoppers. The inside of the " cell " was filled with a borax-boric acid buffer solution and a solution of HCN in a borax buffer was placed in the quinine bottle. It was necessary to use a buffer which would not injure the skin or react chemically with either the cyanide or the silver nitrate solution used to determine the concentration of cyanide. The pH values from 6.8 to 9.2 were obtained by changing

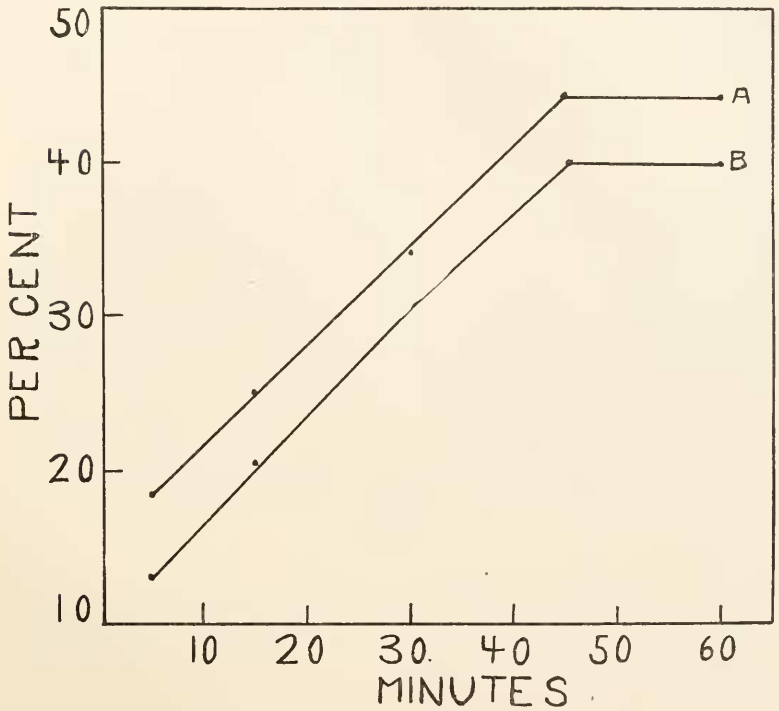

FIG. 1. Curve showing the relation of the position of the skin to the permeability of the skin to cyanide. *A*, skin with the flesh side out; *B*, skin normal, flesh side in. Abscissæ, time in minutes; ordinates per cent. cyanide.

the relative amounts of sodium borate and boric acid; for pH values below 6.8 it was necessary to add small amounts of nitric acid. The addition of HCN did not change the pH of the solution of either the borax or the borax plus nitric acid. Pure liquid hydrogen cyanide was used. No leakage occurred around the skin as shown by the fact that when a " cell " was filled with an

indicator and placed in a dilute solution of HCl the indicator did not change color. Several tests were conducted by reversing the skin and it was noted that the membrane was slightly more permeable to HCN when the skin was turned inside out than when it was in a normal position. The difference, however, was so slight that it is not significant (Fig. 1).

The "cells" after being filled with a borax buffer and put in a solution of HCN, were placed in a water bath at a constant temperature of 25° C. for one hour. At the end of that time equilibrium was reached between the cyanide inside and outside the cell. Five cubic centimeters of the internal and external solution were titrated with $N/50$ silver nitrate, using a one cc. pipette as a burette. The concentration of the cyanide solution used was $M/313$. That the skin was not killed at this concentration was easily demonstrated by substituting for the cyanide solution mineral acids, known not to penetrate living membranes. No change in intracellular acidity was noted in such control experiments. Experiments were conducted using external and internal solutions of various pH values, the external pH varied from 5. to 8.6 and the internal pH from 6.5 to 8.0. The results plotted in Fig. 2, show the relation of concentration of the total cyanide (HCN and CN) found in the cell at equilibrium to the various external and internal pH values. From this figure it may be noted that the penetration curve closely approximates the dissociation curve (x) and that the total concentration of cyanide inside the cell corresponds very closely to the undissociated cyanide in the external solution. The degree of dissociation represented in the curve was calculated from the formula

$$\log 1/H = \text{pH} = \log 1/K + \log a/1-a \quad (19),$$

where a = degree of ionization: K = dissociation constant, for HCN = 4.7×10^{-7} (20).

Figure 2 shows that at equilibrium, when the pH is from 5. to 5.5, the concentration of total cyanide within the cell is equal to the amount of cyanide in the external solution. From the dissociation curve it is evident that at this pH there is practically no dissociation, all of the cyanide being in the molecular condition. As the external pH was increased, the penetration curve rapidly

dropped. Likewise as the pH was increased the degree of ionization was increased. At a pH of 7.0 the dissociation is practically 50 per cent. and the concentration of cyanide in the cell is about 40 per cent. of the concentration of the external solution.

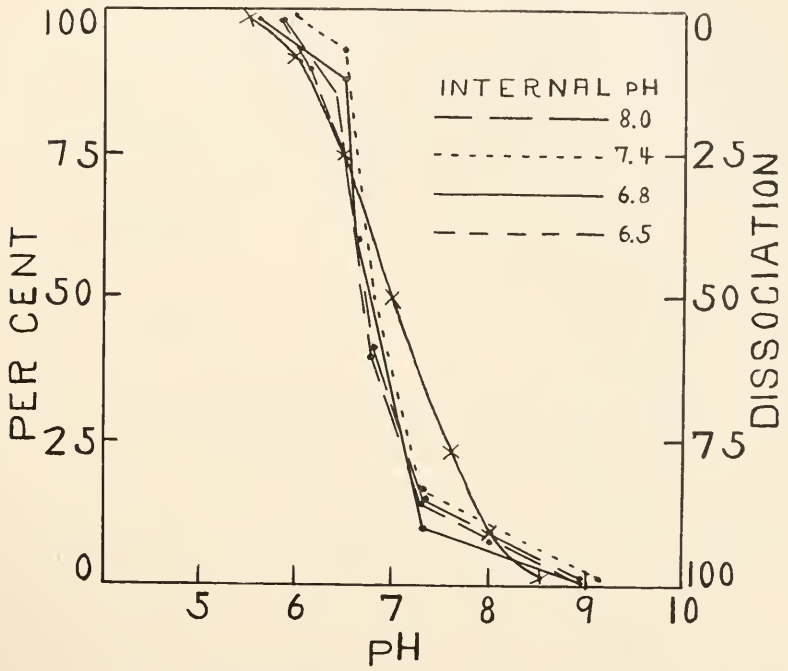

Fig. 2. Curve showing the effect of pH on the permeability of frog skin to HCN. Abscissæ represents the external pH; ordinates the per cent. of total cyanide on the right and the degree of ionization on the left. Calculated degree of dissociations represented by *X*. Internal pH values indicated as follows: short dashes 6.5, solid line 6.8, dotted line 7.4, long dashes 8.0.

At a pH of 9.0 dissociation is practically complete and accordingly little or no cyanide is present in the cell. It is evident that hydrogen cyanide seems to penetrate frog skin chiefly in the form of molecules and not as ions. It is also apparent from Fig. 2 that the intracellular pH varying from 6.5 to 8.0 does not affect the permeability of frog skin to hydrogen cyanide. Brooks (21) stated that changing the pH of the sap of Valonia with CO_2 and NH_3, changed the amount of 2 — 6 — dibromo phenol indophenol

that entered the cells. However, she states and also Scarth (22) shows that the pH of the sap in the vacuole could be no criterion of the pH of the protoplasm.

Experiments with Tadpoles, Daphnia and Elodea.

Experiments conducted with young bull frog tadpoles (Fig. 3) and *Daphnia* (Fig. 4) gave the same relative results as the experiments conducted with cyanide on frog skin "cells." After being separated from their cultural medium by cheese cloth, the organisms were placed in Syracuse watch glasses filled with a solution

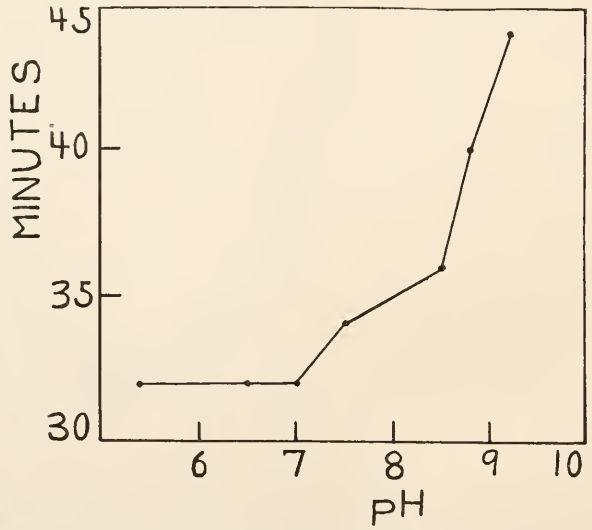

Fig. 3. Curve showing the effect of pH on the toxicity of HCN to young bullfrog tadpoles. Abscissæ represents the pH; ordinates the time in minutes required to produce death. Ten animals were used in each test.

of HCN in a borax buffer solution. It was previously determined that the animals were not killed in the buffer solution free of cyanide until after an exposure of three to four hours. On account of the anesthetic action of the cyanide, tadpoles did not prove to be good material for this work, since it was difficult to determine the time of death. When *Daphnia* was used the beat of the heart could be observed under a binocular microscope and the death point determined at the instant the heart stopped beating. It was noted, that the heart continued beating for some time after

other body movements had ceased and that the organism did not recover after the heart stopped.

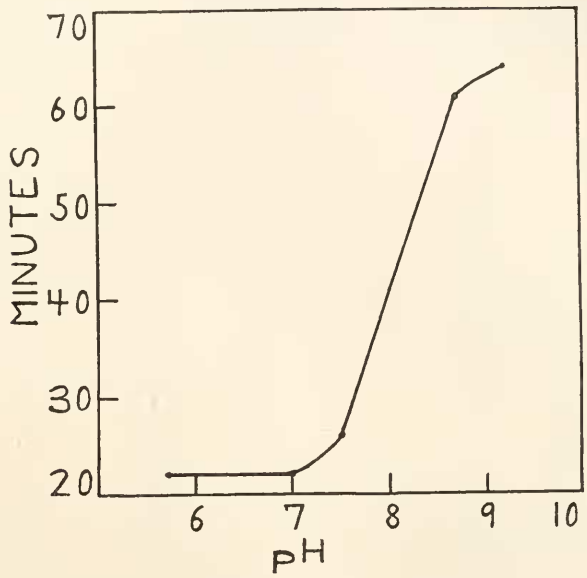

FIG. 4. Curve showing the effect of pH on the toxicity of HCN to *Daphnia*. Abscissæ represents pH; ordinates time in minutes required to kill 95 per cent. of the organisms.

It is obvious from Fig. 3 and 4 that less time was necessary to kill the animals in acid solution than in the alkaline solution. It required twenty-two minutes to kill *Daphnia* at a pH of 5.7 to 7.0; forty minutes at a pH of 8.0 and sixty-four minutes at a pH of 9.0. The concentration of cyanide used in the above experiment was $M/450$. The results indicated what would be expected from the study of the frog skin "cell," where more cyanide entered when the external pH was acid than when alkaline. The animals were killed first in solutions of the same pH values in which HCN penetrated the frog skin cells most quickly.

The effect of the pH on penetration of cyanide was further checked by studying its effect on the streaming of protoplasm in *Elodea* cells (in press). The streaming of the protoplasm can be observed under the high power of a microscope while the cells are immersed in a solution of cyanide. The pH was controlled,

as before, by a borax buffer. Leaves of *Elodea* near the growing tip of the branch were placed in a solution of hydrogen cyanide in Syracuse watch glasses and the time noted when the streaming of the protoplasm ceased. The results of a typical set of experi-

FIG. 5. Curve showing the effect of pH on the streaming of protoplasm in *Elodea* cells. Abscissæ represents pH; ordinates time in minutes required for streaming to stop.

ments are plotted in Fig. 5. Again the results show that the streaming of protoplasm stopped first in solutions of the same pH values in which HCN penetrated the artificial frog skin "cell" most quickly. Thus, in determining the toxicity of hydrogen cyanide to any organism, the value of controlling the hydrogen ion concentration of the solution is apparent.

It is obvious from the foregoing discussion that hydrogen cyanide penetrates living membranes chiefly in the form of molecules and in this respect is similar to other weak acids. Jacobs (18*a*) showed that carbonic acid killed various species of protozoa in a different order than mineral acids, which act primarily through their H ion (Collett, 23). This indicated to Jacobs, that the physiological effect of CO_2 was due to the entrance of the molecule. Beerman (24) and Bodine (25) obtained similar results with H_2S and HCN respectively. More recently, Osterhout (9*b*) and Osterhout and Dorcas (9*c*) showed by direct analysis that hydrogen sulfide and carbonic acid penetrated the living cells of *Valonia* chiefly in the form of molecules and not as ions. Brooks (21*a*) found that the amount of 2 — 6 — dibromo phenol indophenol in the sap of *Valonia* was proportional to the amount of undissociated dye in the external solution.

Effect of Temperature.

A series of experiments was conducted to determine the effect of temperature on the permeability of frog skin to hydrogen cyanide. The results are plotted in Fig. 6. It is evident from these curves that the higher the temperature the greater is the concentration of intracellular cyanide at any stated time. The curve suggests that of a typical unimolecular reaction as can be easily demonstrated by calculating the velocity constant from the following equation:

$K = 1/t \log a/a-x$ (26) in which $x =$ the amount of cyanide in the cell at any time t; $a =$ the amount of cyanide in the cell at equilibrium (Table I.).

Table I.

Velocity Constant K Calculated from the Unimolecular Equation $K = \frac{1}{t} \log \frac{a}{a-x}$ at Various Temperatures.

External and internal pH 6.8.

6°.				16°				25°.			0°.				34.5°.		
t	a	x	k	t	a	x	k	t	a	x	k	t	a	x	k	t	a	x	k
Min.																			
15	1.84	.99	.0222	15	2.37	1.18	.0207	15	3.56	2.24	.0276	15	3.63	1.84	.0204	15	3.69	2.04	.0251
30		.158	.0256	30		2.17	.0357	30		3.11	.0252	30		2.97	.0256	30		2.90	.0223

It will be noted from Table I. that the velocity constant for any temperature is fairly consistent with the exception of 16°, and that K for the various temperatures is nearly constant. Plotting the log of the rate against the reciprocal of the absolute tempera-

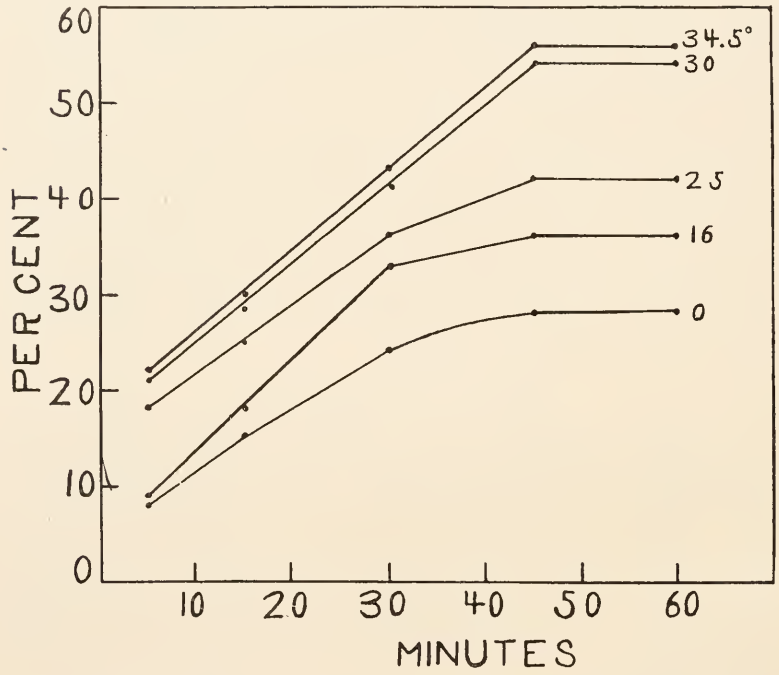

FIG. 6. Curve showing effect of temperature on the permeability of frog skin to HCN. Internal and external pH 6.8. Temperatures used were 0°, 16°, 25°, 30°, 34.5° C. Abscissæ represents exposure in minutes; ordinates per cent. of total cyanide.

ture, a curve was obtained as indicated in Fig. 7. The rate represents the time when the intracellular concentration of cyanide is twenty-five per cent. of the external concentration. It will be seen from this figure that there is a break in the line at 16° C. (.003415). Calculating Q from the Vant Hoff-Arhenius equation, (26)

$$K_2/K_1 = Q/2\left(\frac{T_2 - T_1}{T \cdot T_2}\right),$$

a value of 11,179 is obtained at a temperature from 16° C. to 34.5 and 4,300 for 0° to 16°.

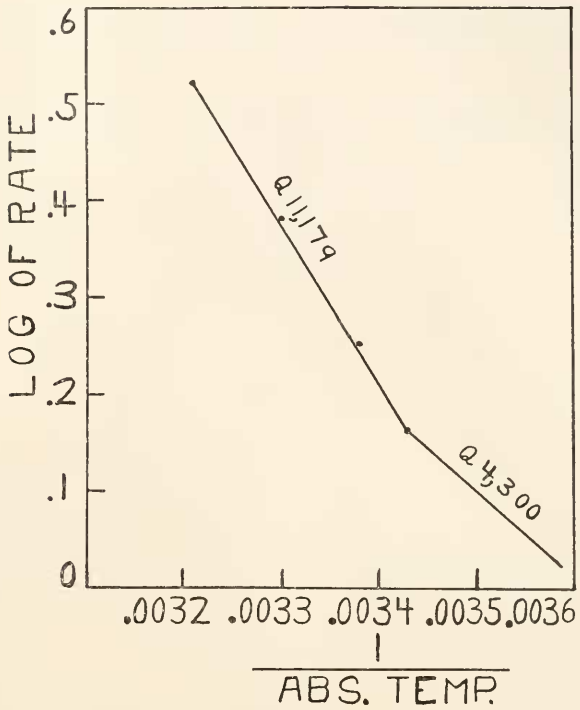

FIG. 7. Curve showing the log of the rate plotted against the reciprocal of the absolute temperature. Rate calculated from Fig. 5 as the time taken for the intracellular cyanide to equal 25 per cent. of the external concentration. Calculated from the formula

$$\frac{K_2}{K_1} = \frac{Q}{2}\left(\frac{t_2 - t_1}{t_2 \cdot t_1}\right).$$

Abscissæ represents reciprocal of the absolute temperature. Ordinates, the log of the rate.

RELATION OF CONCENTRATION.

Experiments were undertaken to determine the effect of the concentration of cyanide in the external solution on the penetration of hydrogen cyanide through frog skin. The external and internal pH and the temperature were maintained constant at 6.8 and 25° C. respectively. The concentrations of cyanide were *M*/109, *M*/124, *M*/160, *M*/196, *M*/225 and *M*/313. The results of such an experiment are plotted in Fig. 8. Each point on the

curve represents the average of three to five tests. It may be seen from this figure that, with the exception of the two low concentrations ($M/225$ and $M/313$), the total amount of cyanide within the cell at equilibrium was the same for all the concentrations. The rate of entrance of cyanide increased with an increase in

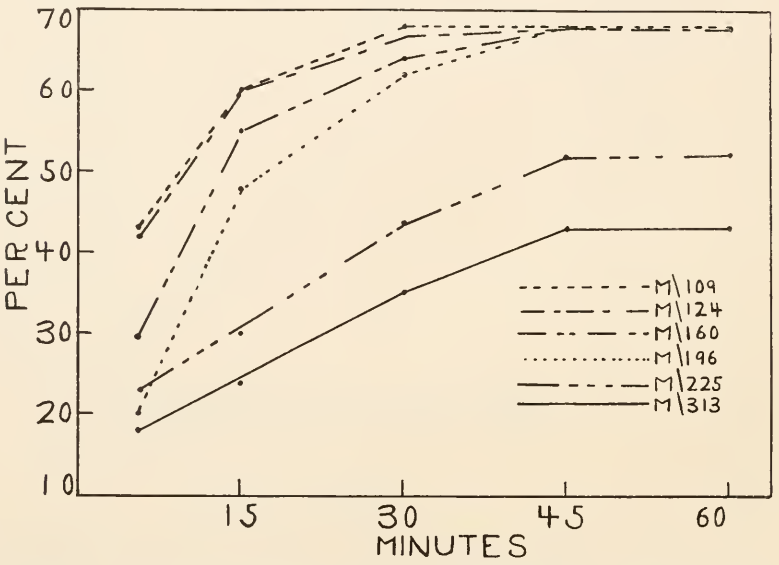

FIG. 8. Curve showing the effect of concentration of HCN upon the permeability of frog skin to cyanide. Concentrations of HCN used were $M/109$, $M/124$, $M/160$, $M/196$, $M/225$, and $M/313$. Abscissæ represents the time in minutes; ordinates per cent. cyanide.

concentration. At a concentration of $M/109$, equilibrium was reached within 30 minutes, while at lower concentrations equilibrium was not reached for a period of 45 minutes. The fact that the skin is not killed can be proved by substituting for the cyanide solution a mineral acid, which is known not to pass through living membranes, and testing the pH of the internal solution. As there is no change in the intracellular acidity, it is evident that no acid has passed through the skin.

Although, it is known that the frog skin is not killed by the above treatment with HCN, it is desirable to ascertain what effect the cyanide does have upon it. A series of experiments was conducted to determine the effect of hydrogen cyanide on the po-

tential difference of frog skin and the results are given below in detail.

Effect of Hydrogen Cyanide on the Potential Difference of Frog Skin.

Osterhout (*9c*) and others have shown that the electrical resistance of an organism is an excellent indicator of its vitality and that death is accompanied by an increase in permeability. An increase in permeability is equivalent to an increase in the electrical conductivity or to a decrease in resistance. In order to determine the physiological effect of hydrogen cyanide on frog skin, it was

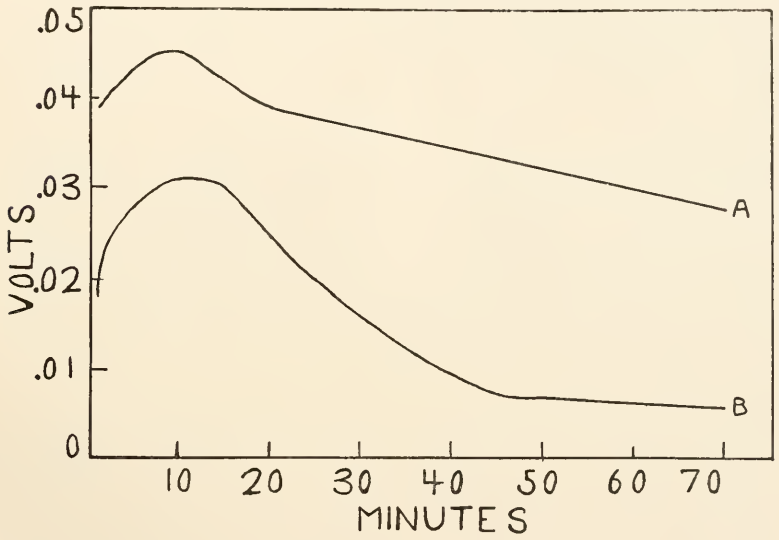

Fig. 9. Control experiments; curve showing the P.D. of frog skin in *A*, Ringer's solution and *B*, borax-boric acid buffer. Abscissæ represents the exposure in minutes. Ordinates P.D. in volts. Different pieces of skin from the same frog were used.

potentiometer. The same solution was placed inside and outside the cell. The skin from the hind legs of bull frogs (*Rana cates-* thought advisable to study its potential difference before and after being immersed in various solutions of this chemical. The apparatus was similar to that previously used in the first part of this paper with the exception that non-polarizable zinc electrodes were placed inside and outside the cell and the potential read on a

biana) was used. Since the potential difference of the skin from different frogs and also different pieces of skin from the same frog, varied considerably, it was necessary to repeat each experiment many times and only characteristic curves of single experiments will be given. There is some evidence at hand to indicate that the conditions under which the frogs are kept influences to some extent the potential difference of the skin. It was observed in several instances, when the temperature of the vivarium dropped several degrees below normal, that the potential difference of the skin also dropped and that on warm days the potential difference was usually higher than that at other times.

Control experiments were conducted by placing the skin in solutions of borax-boric acid buffer and Ringer's solution. The result of a typical control test is given in Fig. 9. Readings on the potentiometer were taken every minute. The temperature varied during the tests from 20–21° C. The experiments were run in parallel series using different pieces of skin from the same animal. It will be noted from Fig. 9, that there was an initial rise in P.D. followed by a gradual decline. The two curves are approximately parallel indicating that the borax buffer is no more toxic to frog skin than is the Ringer's solution. The pH of the Ringer's solution was about 8.2 and the borax 6.8.

A series of experiments was undertaken to determine the relation of the concentration of cyanide to the potential difference of frog skin. The cyanide solution was made by adding pure liquid HCN to a borax buffer at a pH of 6.8. The concentrations of cyanide used were $M/136$, $M/154$ and $M/225$. Fig. 10 shows the results of a typical set of experiments. The cells were placed in a borax buffer for ten minutes, then removed and placed in the cyanide solution. The skin was allowed to remain in the cyanide solution for various periods of time, then removed and placed in a borax buffer free from HCN. The period between arrows indicates the time that the cells were exposed to cyanide. In all cases it may be observed that the skin completely recovered after being removed from the cyanide solution. After the cells had been removed from the borax solution and placed in cyanide solution at a concentration of $M/225$, a great stimulation occurred, followed by a gradual drop in the potential difference to the base line. This

stimulation, characterized by a rise in potential difference, is also evident at a concentration of $M/154$ but not nearly to the same degree as with the weaker concentration of cyanide. The drop following stimulation at a concentration of $M/154$ is practically of the same magnitude as obtained with a $M/225$ solution. In the case of the $M/136$ solution there was no stimulation but the potential difference dropped suddenly to the base line. The skin

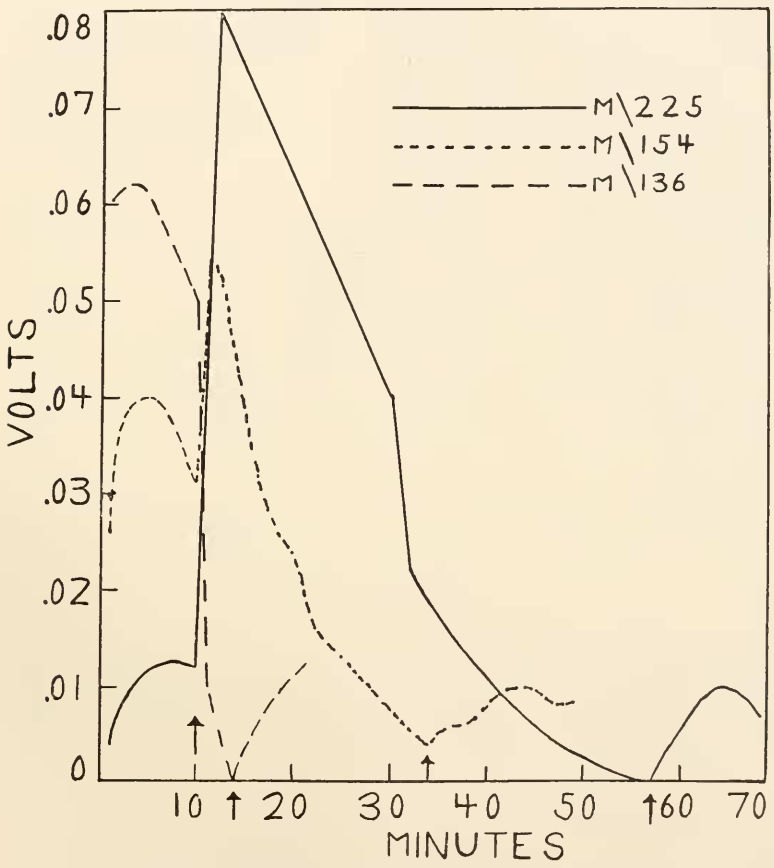

FIG. 10. Curve showing the relation of concentration of HCN to the P.D. of frog skin. Concentration of total cyanide = $M/225$, $M/154$ and $M/136$. Same solution on both sides of the membrane. The period between arrows indicates the time that the skin was exposed to cyanide, at other times the skin was exposed to borax buffer free from HCN. Abscissæ represents the time in minutes; ordinates the P.D. in volts. Different pieces of skin from the same frog were used.

recovered when removed from the cyanide. It is obvious from Fig. 10, that a weak solution of cyanide acted at first as a stimulant and this was followed by a delayed toxicity. As the concen-

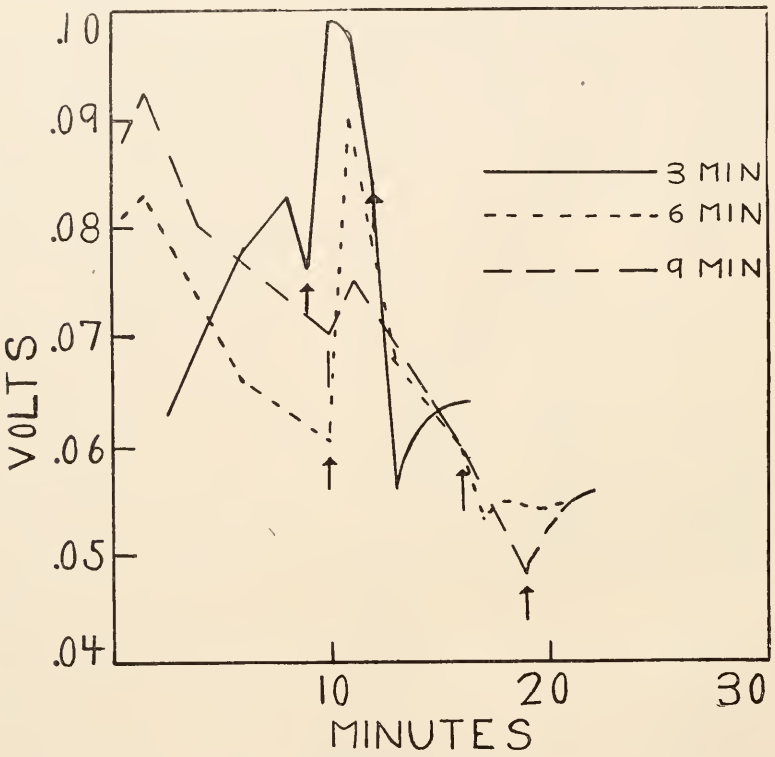

FIG. 11. Curve showing the effect of short exposures of cyanide to the P.D. of frog skin. The time between arrows represents the period of exposure to HCN, at other times the skin was exposed to borax buffer alone. Solid line represents a three minute exposure to cyanide; dotted line, 6. min. exposure and broken line 9 min. exposure. Abscissæ represents the exposure in minutes; ordinates, the P.D. in volts.

tration of cyanide was increased, the stimulation decreased and the toxic effect increased until a point was reached where stimulation no longer occurred and at this point the toxic action was pronounced. This initial stimulation of frog skin by cyanide was apparently overlooked by Lund (*7a*) who took readings every fifteen minutes. He did notice, however, a rapid rise followed by a rapid fall in the electrical resistance in Obelia during a period

of exposure to KCN. It is well known that many anesthetics in dilute solutions act as protoplasmic stimulants and in concentrated solutions their toxic action is established (Osterhout, 9).

It is interesting in this connection to determine whether the rapid fall in the potential difference immediately following stimulation by dilute cyanide is due to a natural recovery to normal or to the toxic action of the cyanide. Cells were removed from the borax buffer solution at the end of a ten minute exposure and placed in a hydrogen cyanide solution of a concentration of $M/160$. A series of three experiments was conducted; in the first series the cells were removed from the cyanide solution at the end of three minutes and placed in a borax buffer, at which time the stimulation had reached its maximum and started to drop. In the second series the skin was allowed to remain in the cyanide solution for six minutes, then removed and placed in the pure buffer, at which time the drop following stimulation had reached the same reading as when the skin was first placed in the cyanide. In the third series the skin was exposed to the cyanide for a period of nine minutes. At the end of that time, the drop following stimulation had reached a point below the original P.D. The results of a typical series of experiments are plotted in Fig. 11. It is evident from this figure that after the removal of the skin from the cyanide solution, in the three and six minute exposure, the drop in P.D. continued until it had fallen below the P.D. obtained at the time the skin was placed in the cyanide solution; the P.D. then increased to normal. The nine minute exposure showed no further drop in the P.D. after being removed from the cyanide solution but an immediate recovery occurred. It is apparent from the data given that the drop in potential difference immediately following the stimulation was due not to the toxic action of the cyanide but to a natural return to the original reading and the toxic action did not take place until after the drop had surpassed the point where the skin was stimulated.

Effect of the pH of Cyanide Solution on the Potential Difference.

It is apparent from the data given in the first part of this paper that little or no HCN penetrates living membranes except in the

form of undissociated molecules; thus it was deemed advisable to study the effect of dissociated and undissociated molecules of HCN on the potential difference of frog skin. Control experiments were conducted using borax-boric buffer free from cyanide at a pH of 6.8 and 8.5 to determine whether or not the hydrogen ion has any effect on the frog skin. Fig. 12 shows the results

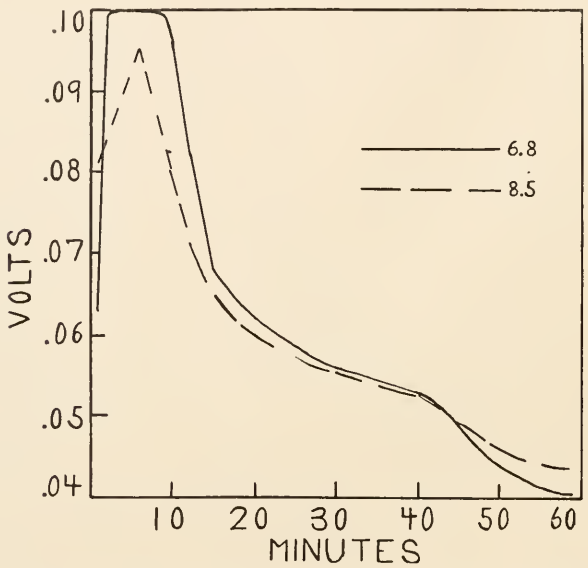

Fig. 12. Curve showing the effect of pH of the borax buffer on the potential difference of frog skin. pH valves 6.8 and 8.5. Abscissæ represents the exposure in minutes; ordinates, the P.D. in volts.

obtained. It is obvious that the two curves coincide very closely and that the hydrogen ion had no effect on the skin. It may be noted that the curves are strikingly similar to the control curves plotted in Fig. 9. The initial rise followed by a gradual decline was again manifested. Fig. 13 shows the results obtained by placing the frog skin "cells" in a solution of HCN in borax buffer at pH values of 6.8 and 8.5. The concentration of cyanide used was M/154. The temperature was constant at 22° C. ± 0.5°. The "cells" were placed for ten minutes in a borax buffer solution at pH values of 6.8 and 8.5 respectively; at the end of that time they were removed and put in a borax buffer containing

HCN at their respective pH values. The period of exposure to was greater at a pH of 8.5 than at 6.8. It is also apparent that the drop in the P.D. after a return to normal was somewhat more cyanide is represented on the curve by the time between the arrows. From Fig. 13, it is obvious that the initial stimulation pronounced under the acid conditions than under the alkaline.

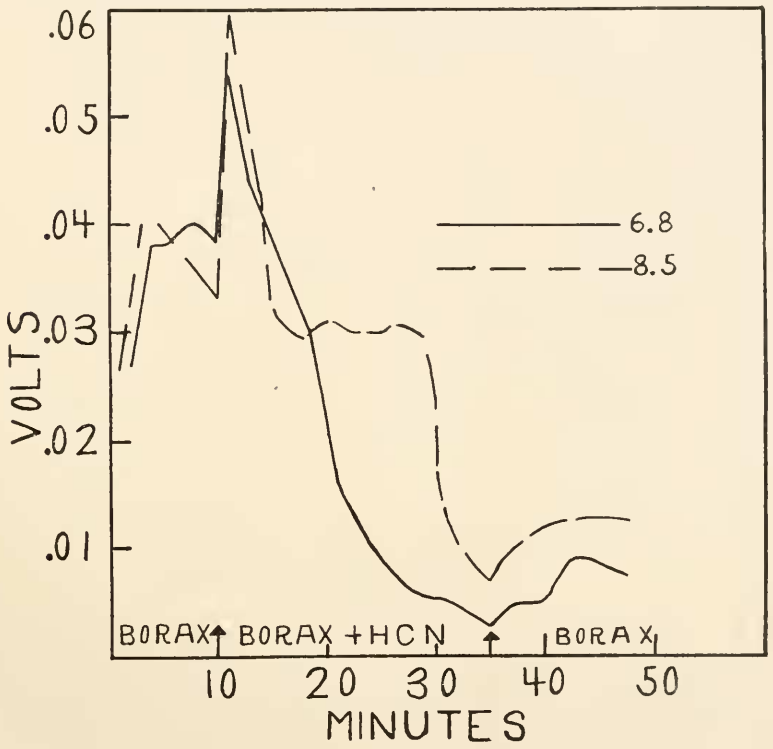

Fig. 13. Curve showing the effect of the pH of a solution of HCN in borax on the P.D. of frog skin. Period of exposure to cyanide represented as the time between arrows, at other times the skin was exposed to borax alone. pH values used were 6.8 and 8.5. Abscissæ represents the exposure in minutes and the ordinates the P.D. in volts.

There actually are fewer molecules of cyanide in a solution with a pH of 8.5 than with a pH of 6.8 (Fig. 1). So if the molecules were the toxic units, it would be expected that there would be less stimulation and more toxicity when the solution contains the greatest number of molecules than under the reverse conditions of

less molecules and more ions. Such a condition was found to exist, as is evident from Fig. 13 where more stimulation and less toxicity occurred under the alkaline conditions than in the acid solutions.

In Fig. 14 are plotted the results of a series of tests using a solution of HCN in Ringer's solution. The pH of Ringer's solution was about 8.2; by the addition of HCl the pH was changed to

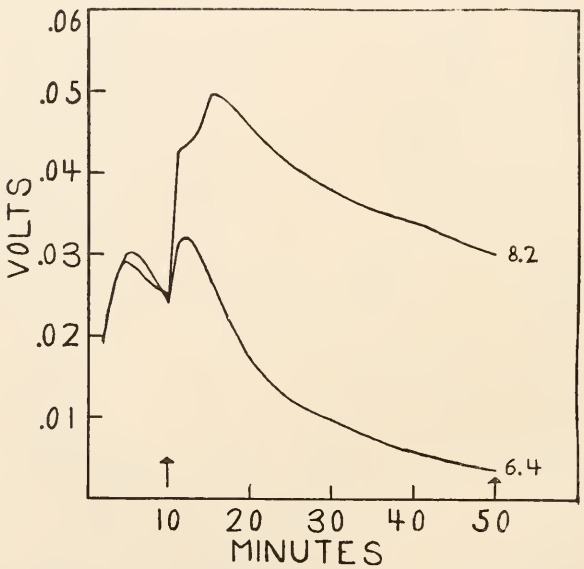

FIG. 14. Curve showing the effect of the pH of a solution of HCN in Ringer's solution. Exposure to cyanide represented by the time between arrows. First ten minutes skin exposed to Ringer's solution alone. pH values used were 6.4 and 8.2. Abscissæ represents the exposure in minutes and the ordinates the P.D. in volts.

6.4. The same concentration of cyanide was used in each series ($M/250$). The "cells" were placed in Ringer's solution for ten minutes, then removed; one series was placed in a solution of HCN in Ringer's solution at a pH of 6.4 and the other series at a pH of 8.2. Potentiometer readings were taken every minute for 45 minutes. Again it is evident from Fig. 14, that there is greater stimulation and less toxicity under alkaline than acid conditions. In either case the initial stimulation was not as pronounced as in the experiments when borax buffer solution was used. It is, how-

ever, sufficiently marked to be significant. Figs. 15 and 16 show the results obtained when the pH was alternated from 6.6 to 8.2 and vice versa. The same piece of skin was used under the same conditions of temperature and concentration of cyanide; the concentration of cyanide was $M/173$, made by adding liquid HCN to

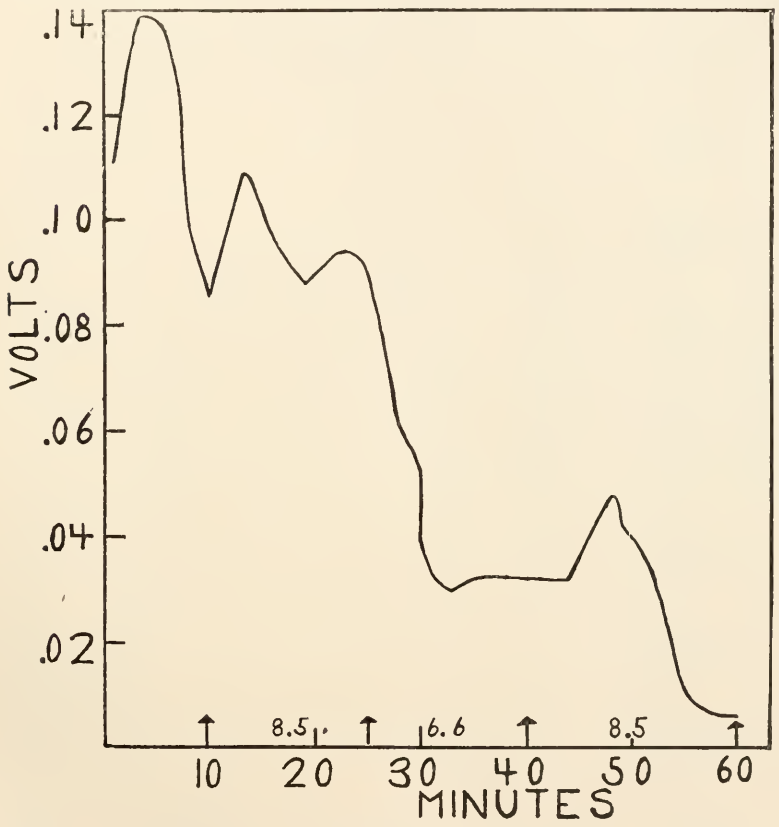

FIG. 15. Curve showing the effect of alternating the pH of a solution of HCN in borax on the same piece of frog skin. pH changed from 8.5 to 6.6 then back to 8.5. Period of exposure at various pH value indicated in curve. For the first ten minutes the skin was exposed in minutes and the ordinates, the P.D. in volts.

a borax buffer. The skin was placed in a borax solution for ten minutes, then removed and placed in the cyanide solution at a pH of 8.5 for 15 minutes (Fig. 15). The characteristic curve was obtained—an increase in P.D. followed by a gradual decrease.

When the period of 15 minutes had elapsed, the skin was removed and placed in a HCN solution of the same concentration and at a pH of 6.6. It will be noted from Fig. 15 that a very sudden drop occurred in the P.D. as soon as the skin was placed in the solution at a pH of 6.6. At the end of 15 minutes the skin was again placed in the original HCN solution at a pH of 8.2.

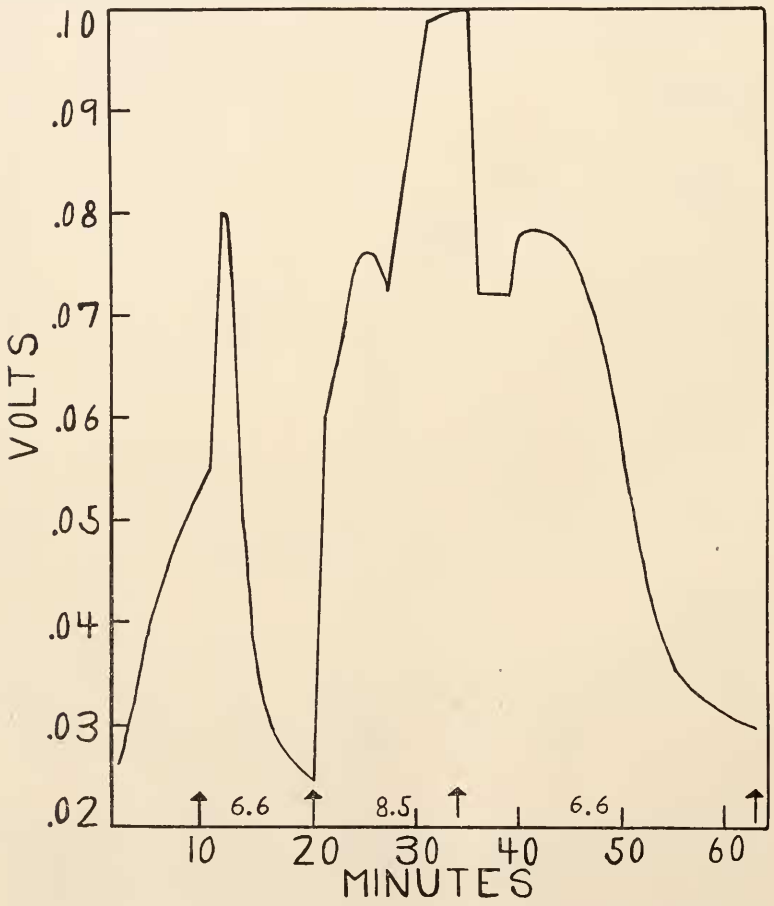

FIG. 16. Curve showing the effect of alternating the pH of a solution of HCN in borax on the same piece of frog skin. PH changed from 6.6 to 8.5 then back to 6.6. Period of exposure at various pH values indicated in curve. For the first ten minutes the skin was exposed to borax alone. Abscissæ represents time of exposure in minutes and the ordinates the P.D. in volts.

The P.D. gradually increased—suggesting a recovery—followed by a gradual decrease to the base line. The curve suggests, that at a pH of 6.6 the cyanide was more toxic than at a pH of 8.2 as was evident from the fact that the P.D. suddenly dropped when the skin was taken from a solution of a pH of 8.2 and placed in a pH of 6.6. When the skin was taken from a pH of 6.6 and placed in a solution of 8.5 a recovery occurred.

The conditions plotted in Fig. 16 are the reverse from those in Fig. 15. The skin after being placed in a borax buffer solution for ten minutes was removed and put in a cyanide solution of a pH of 6.6. The initial rise followed by a rapid drop was again obtained. When, however, the skin was removed from the pH of 6.6 and placed in a cyanide solution having a pH of 8.5, the P.D. rapidly increased until a point was reached above the initial stimulation suggesting a recovery and a stimulation. At the end of 15 minutes the skin was again placed in the original HCN solution at a pH of 6.6; a rapid fall occurred, suggesting a return to normal, followed by a rapid decline. As previously stated, 98 per cent. of the total cyanide is dissociated at a pH of 8.5 and thus the cyanide is present in the ionic condition. When the pH is 6.6 the cyanide is about 40 per cent. dissociated, therefore, the number of molecules and ions is about equal. From the data given, it appears that the cyanide is more toxic to the frog skin when the solution is acid than when alkaline. Thus, it seems that the molecule is actually more toxic than the ion. In dilute concentrations the physiological effect of hydrogen cyanide on frog skin is first a stimulation followed immediately by a rapid return to the original reading terminating in a toxic effect which will eventually prove fatal.

Summary.

The experiments indicate that little or no HCN penetrates frog skin "cells" except in the form of undissociated molecules. The total amount of intracellular cyanide is proportional to the concentration of undissociated molecules in the external solution. The internal pH value of the "cell" has no effect on the penetrations of HCN through frog skin.

From a study of the effect of hydrogen cyanide on the potential

difference of frog skin, it appears that dilute solutions of cyanide cause an initial stimulation followed by toxicity. As the concentration is increased, the stimulation is decreased and the toxicity is increased until a certain concentration of cyanide is reached where there is no stimulation but a marked toxic effect is evident.

The data also indicate that the molecule is more toxic than the ion.

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SEX RATIO IN *GAMBUSIA*.[1]

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INTRODUCTION.

The unequal division of the sexes in adult *Gambusia* and other viviparous minnows is a subject discussed by many writers. The present author has had an opportunity, in connection with the study of the value of *Gambusia* as an agent for the control of mosquitoes, to make many observations and to sex numerous fish consisting of both adults and juveniles and the results from this work form the basis for the present paper.[2] The material upon which the present study was based was obtained chiefly in three localities, namely, Augusta, Ga., Beaufort, N. C., and Greenwood, Miss. Therefore, the data, according to the most recent classification (Geiser, 1923, and Hubbs, 1926), are based on two species of *Gambusia,* namely *holbrooki* from Augusta and Beaufort, and *affinis,* according to Geiser, or *patruelis* according to Hubbs, from Greenwood. The present writer is unable to discuss the merits of the further division between *affinis* and *patreulis,* recently recognized by Hubbs. However, the differences in the structure of the distal part of the anal fin of adult males from Augusta and Beaufort (Atlantic drainage) and those from Greenwood (Mississsippi River drainage) are unmistakable and they are correctly described and figured by Geiser (1923). Furthermore, the present writer finds that when the rays in the dorsal fin are accurately and uniformly enumerated in both species, that is, when the last two rays that are more or less united, are counted either as one, or as two rays, a constant difference of one ray becomes evident.

[1] Published by permission of the U. S. Commissioner of Fisheries.

[2] The writer wishes to acknowledge the very valuable assistance rendered by Irving L. Towers, recently junior aquatic biologist, U. S. Bureau of Fisheries, who was associated with the author for several years in the *Gambusia*-mosquito studies and who personally sexed many adult fish and made numerous observations herein recorded.

When counting the last, partly united, rays as two in 81 specimens examined from the Atlantic drainage (Augusta, Ga., Beaufort, N. C., Orangeburg, S. C., and Key West, Fla.) the dorsal fin constantly had 8 rays. Similarly, in 78 specimens examined from the Mississippi River drainage (Greenwood, Miss., Memphis, Tenn., Mound, La., and Little Rock, Ark.) 7 dorsal rays invariably were present. The variation of two and three rays reported in published works, therefore, probably is due chiefly to a difference in enumeration. The difference in the number of dorsal rays, then, is helpful in separating the otherwise bothersome females, as well as all juveniles which have not yet developed the external sexual characters.[1]

Although the data, forming the basis for the present paper, are founded upon two species of *Gambusia,* they will not be delt with separately, as no differences between the species with respect to sex ratio, neither seasonal, for adults, nor for young fish were noticed. The specimens, collected from 1921 to 1926, were taken in a large variety of waters, that is, ponds of various sizes (with and without vegetation) borrow pits, swamps (both fresh and brackish), ditches and sluggish creeks. The specimens, in fact, were taken in nearly every environment in which *Gambusia* lives. The data, therefore, are representative of animals living under a large variety of conditions, and not of any particular environment.

Although many authors, as already stated, have discussed the sex ratio in adult Pœciliids, little work has been done on the sex ratio of immature fish. The only account of the sex ratio of young Pœciliids, of any importance, that has come to the writer's notice is the one by Geiser (1924). Even Doctor Geiser's data are rather limited, yet they show quite accurately the sex ratio of young *Gambusia,* as will be demonstrated by the extensive data that are offered in a subsequent section of this paper.

[1] It appears to be of interest to note here that a small collection (26 specimens) from Camilla, Ga., is at hand in which occur five females with only 7 rays in the dorsal fin, whereas all the others, including two males, have 8 rays. Geiser (1923) studied specimens from the same collection and, basing his identification on the minute structure of the intromittent organ of adult males, found them to represent the eastern form, *holbrooki,* although Camilla is in the Gulf drainage. It would now appear as if both species occur in this locality. Unfortunately no males with 7 dorsal rays are at hand with which to verify this identification.

Sex Ratio in Adult *Gambusia*.

The literature dealing with the sex ratio of adult Pœciliids, as previously indicated, is extensive and scattered and it will not be reviewed here. The reader, however, is referred to Dr. Samuel W. Geiser's admirable paper, "Sex-ratio and Spermatogenesis in the Top Minnow, *Gambusia holbrooki* Grd.," (1924) for a review of this literature and a concise discussion on the subject. It is sufficient to say here that the inequality of the sexes in adult fish is an unmistakable and a positive fact. As further proof, Table I., based on 103,150 fish, examined for sex, is offered. In explanation, it may be stated that the fish were collected either with a bobbinet seine or a dipnet made of the same material. Therefore, it was impossible for the smaller males to escape through the meshes of the nets.

In general fish having a total length of about 21 mm. (using "eye measure") and over were considered adult fish and are included in Table I. The modified anal fin, serving as a copulatory organ, of course, was used in separating the sexes. That is, when a fish possessed a modified anal fin it was classed as a male, otherwise as a female. Many males, as will be explained later, undoubtedly were classed as females, because of the late development of the copulatory organ in some individuals. Occasionally this organ is fully developed when the fish is 18 mm. long and generally if not fully formed it is at least sufficiently developed to admit of recognition when the fish is about 21 mm. long. However, many exceptions are found, as is shown in the section of this paper dealing with the sex ratio of immature fish. It is enough to say here, that the disparity of the sexes, that is, the minority of males, is not as great as shown in Table I. The number of males, classed as females, nevertheless, is entirely too small wholly to account for the large inequality in the sexes, and the fact that the males are greatly in the minority among adult fish unquestionably remains.

It was shown by Barney and Anson (1921) that a "seasonal male frequency" takes place. The data presented in Table I. bear out the information offered by these writers, namely that the males are comparatively much more numerous during the fall,

winter and spring than they are in midsummer. The data at hand are for the following months: June, July, August, September, October and December. Males were most numerous, as shown by these data, in June, when the ratio was 1 male to 2.54 females among 4,902 fish examined. In July the ratio, based on the examination of 17,941 fish, was 1 male to 5 females. In August the males were the fewest, as a ratio of only 1 male to 11.3 females obtained, as shown by the examination of 21,446 fish. In September the males began to increase, for a ratio of 1 male to 8.35 females was obtained from a total of 13,473 fish. In October the males increased still further, for 1 male to 2.75 females was found among 43,288 fish examined. A single day's collection, from Beaufort, N. C., taken chiefly in brackish swamps, for December is at hand, which consists of 2,100 fish. The sex ratio in this collection consists of 1 male to 3.6 females. The number examined obviously is too small and the environment too limited to be representative of the usual sex ratio prevailing at this season of the year. The average ratio of males to females for all fish (103,120) examined is 1 to 4.4.

The comparatively large seasonal difference in sex ratio in *Gambusia,* is very interesting. Barney and Anson (1921) offer the following explanation (pp. 64 to 66): "Further inquiry into the causes of the varying seasonal frequency of males and of the species, shows that in the summer there is a decrease in depth and area of all water systems studied. . . . This lowering of the water in the bayous and ponds eliminates all the shallow margins which *Gambusia* frequent and where they are largely immune from their enemies, and forces them into deeper water where the incline of the banks is steeper. This accordingly means much less natural protection than the minnows formerly had, since predaceous fish, now at a period of heightened metabolism and consequent rapid growth, are especially destructive and have a marked effect in lowering *Gambusia* frequency. Doubtless the *Gambusia* eaten by their predators at this time are nearly all females of large size, since the adult females, gravid at this season, are much larger, much slower in movement, and are more noticeable because of their black abdominal spots than the small, quickly moving, uniformly colored male. With the height of *Gambusia* frequency

in late August (1919) occurs properly the height of the young *Gambusia* frequency. The young frequency then diminishes quickly, apparently due to the scarcity of breeding males during the spring and summer, and the resulting lowered birth rate. The height of *Gambusia* frequency is immediately followed by a considerable drop in water stage (surface water index) and accompanied by an increase in the percentage of other species, many of which are predators. The *Gambusia* frequency decreases by loss of adult females, while the male frequency accordingly increases." In the light of the present studies, as well as the tables presented by Barney and Anson (1921) this explanation is entirely inadequate, and in the main contrary to what actually takes place. If, in the thinning out process, more females than males were eliminated, then, certainly, there should be an abundance of males in July and August. That is, at a time when few young of the current season have become sexually mature and when it is usually quite possible to distinguish between the young of the current season and those of the previous year. Barney and Anson's table, as well as Table I. (presented herewith), however, show that the reverse unmistakably is true. Furthermore, it may be remarked here that the error in sexing, by means of external characters, very probably is smaller during July and August than at any other time. That is, fish of the previous season have become sexually mature and comparatively few young of the current season have reached a sufficiently large size to be considered adults. Therefore, comparatively few males, at this time, are classed as females.

How may the seasonal variation in sex ratio of *Gambusia* then be explained? It is shown in a subsequent section of this paper that males and females are equally proportioned among young *Gambusia,* and it already has been stated that a very large decrease in the proportionate number of males present takes place shortly before (July and August) the main body of young fish of the current season become sexually mature. A greater thinning out of adult males than of adult females, therefore, must have taken place. A discussion of the evidently much more rapid decrease among the males than among the females, when few young fish are maturing to take their place, is reserved for a later section. It appears to be sufficient to state here that the proportionately

greater abundance of males which begin to appear in September are the result of young fish that are becoming sexually mature, for among these fish the males are quite as numerous as the females. After the following May or June nearly all of the fish, born the previous year, have become sexually mature and the young of the current season are still nearly all immature fish. Then the very rapid decline in the male population, already mentioned, takes place.

Further study has convinced the writer that the main reproductive season of *Gambusia* is much more limited than stated by him (1917 and 1921). In catching and transporting large numbers of fish while engaged in the study of *Gambusia* in its relation to mosquito control, it became evident that, although some gravid females are seen in early spring and during the fall,[1] the main reproductive season lasts only about three months, namely June, July and August. A very sudden decrease in the number of gravid females was noticed at Augusta, Ga. (where special attention was given to this matter for several years in succession) during the early days of September and in that locality, at least, the reproductive season may be said to end by Labor Day. The end of the spawning period evidently is not greatly influenced by temperature, as has been the contention of various writers, for the month of September at Augusta generally is one of the warmest of the summer. Furthermore, the writer has for two winters (1925–26 and 1926–27) kept two lots of adult *Gambusia,* containing a fair percentage of males, in the terrapin nursery house at the U. S. Fisheries Biological Station, Beaufort, N. C., which is kept at summer heat. Yet reproduction was not induced during the winter months. It was noticed, however, that gravid females appeared somewhat earlier in the spring in the lots in the terrapin house than among fish that wintered out of doors. The influence of temperature on reproduction in *Gambusia,* therefore, does not appear to be very pronounced.

The scarcity of gravid females in "midsummer" was noticed by Barney and Anson (1921). These authors thought this to be due to the scarcity of males, stating that such a "condition would

[1] In the extreme southern parts of the United States, as at Key West, some gravid females are present at all seasons of the year.

not normally occur in midsummer with a proper relative number of males present." These authors probably overlooked the fact that copulation is not necessary between broods in *Gambusia,* nor in certain other Pœciliids, as it has been shown by Zolotnisky (1901), Philippi (1908) and Hildebrand (1917) that several broods of young may be produced by females after they are separated from males. For example, Hildebrand (1917) segregated some female *Gambusia* early in the spring, providing them with individual aquaria. One female, at least, produced five broods of young that summer, without further copulation. If copulation is not necessary during the summer, as appears to be the case, then certainly the scarcity of males cannot account for the cessation of reproduction during midsummer. Furthermore, the writer has held both sexes of *Gambusia* together in aquaria for a number of years and he has not noticed that the reproductive period is lengthened thereby. It appears to be logical, therefore, to conclude that the great reduction in the proportionate number of gravid females at the end of August is not due to the scarcity of adult males. On the contrary, it is the opinion of the writer that the animals by that time have expended all of the energy on reproduction they can afford and breeding ends, regardless of weather conditions.

TABLE I.

THE SEX IN ADULT *Gambusia.*

Month.	Males.	Females.	Ratio.
June	1,385	3,517	1 : 2.54
July	2,960	14,981	1 : 5.06
August	1,742	19,704	1 : 11.31
September	1,440	12,033	1 : 8.36
October	11,614	31,674	1 : 2.75
December	455	1,645	1 : 3.6
Total	19,596	83,554	1 : 4.4

SEX RATIO IN IMMATURE *Gambusia.*

It already has been pointed out that the sex ratio in young fish has received comparatively little attention. This is rather surprising in view of the many accounts that have been published dealing with the sex ratio of adult Pœciliids. Perhaps this interesting problem did not receive more attention for the want of

proper material and because of the difficulty involved in determining the sexes in young fish. Sexing immature fish, however, was found easier than the writer had anticipated, and a brief description is offered of the technique pursued.

Sexually mature fish at first were dissected with the view of learning definitely the exact position of the gonads and to study the general appearance of these organs in fish in which the sex was definitely known. It was found that with scissors the tail and a part of the back of the fish might be removed by a single clip and by making this cut through the base of the anal and sloping it forward at an angle of about 45 degrees the viscera, after removing the peritoneum, was exposed while remaining intact. The sexual organs, lying dorsally of the visceral mass, may then be examined in position or removed for examination. It will be noticed at once that the ovary has a black membranous covering, whereas, the testes in preserved specimens are pale in color. The testes, although lying very close together and described as " fused " by Geiser (1924), nevertheless, show distinctly a median depression and on this line they may be separated with a sharp needle into two nearly equal parts. The ovary, on the other hand, is definitely fused, it has no median line of depression and cannot be separated into equal parts without causing rough, unequal breaks. The more prominent projection into the abdominal cavity of the interhæmal spines in the male is another difference that is usable. These characters can be used in sexing immature fish from 15 mm. and upward in length, as they are evident under a binocular microscope.

In sexing fish that are less than 15 mm. long it is necessary to be more careful in removing the minute gonads and for differentiation they must be placed on a slide. As it is necessary to use transmitted light, the contents have to be spread. This may be accomplished by placing a cover glass over the glands and tapping it lightly. It is desirable at first to mount and study the gonads of somewhat larger fish, that is, of fish large enough to admit of sex determination by the method described in the preceding paragraph. This is desirable, because confusion may arise from the fact that the sperm cysts might be regarded as eggs. It will be noticed, however, under proper magnification that the cysts are more

opaque and granular in appearance than the ova and they do not have a central "nucleus" which is quite characteristic of the eggs. It is possible to sex *Gambusia* at birth, that is when only about 8 mm. long by this method. Consequently, no tedious processes of embedding and sectioning of gonads need be involved. After a little practice one becomes quite proficient in removing the organs, as well as certain of the diagnosis.

It of course is understood that the specimens, especially the very small ones, must be well preserved or sexing by the process described becomes difficult and unreliable. The best results were obtained by the use of 80 per cent. alcohol to which 40 per cent. formaldehyde was added in the proportion of about 30 cc. to 400 cc. of the alcohol. The strength of the preservative of course may be considerably varied according to the temperature. During cool weather 80 per cent. alcohol alone preserves specimens quite as well as the mixture of the two, in the proportions given, during hot weather.

The rapidity with which immature fish could be sexed, in the manner described, made it possible to examine a comparatively large number of fish, as shown in Table II. Few males have become sexually mature at a length of 20 mm. and no appreciable change in the ratio of males to females has taken place in fish of this size. This is brought out in Table II., in which fish, 20 mm. and under in length, are divided into two groups, namely those of 15.5 to 20 mm. being placed in one group and those of 15 mm. and under in another. It will be seen that in each group the males and females are very nearly equally numerous. This seems also to show that no thinning out of males takes place before they have become sexually mature.

The data for the next group consisting of larger fish (20.5 to 25 mm.) listed in Table II. are not representative of the sex ratio in unselected fish of that size. It is understood that many fish 20.5 to 25 mm. in length are sexually fully mature, the males being recognizable by the modified anal fin and the females, if not actually gravid, nevertheless, frequently have a small elongate blackish spot or line in the position where the prominent black spot is situated when the abdominal walls are distended with eggs and young. Such fish are not included in this group, as it is based

only on specimens that had developed no external sex characters by means of which they could be definitely recognized. These data show that no hard and fast line, with respect to size, can be drawn between adult and immature fish, as the external sex characters are developed at a much larger size in some individuals than in others. In this group of large immature fish, based on 1,660 specimens, the males, however, are considerably in the minority, as the ratio is 1 male to 1.48 female.

A few immature fish of even a larger size than the group discussed in the preceding paragraph were found. For example, among 285 fish, ranging from 25.5 to 30 mm. in length which could not be sexed definitely from external characters, the ratio was 1 male to 6.12 females. In this connection it may be noted, however, that not a single immature male exceeding a length of 28 mm. was found.

The variation in size and age at which the anal fin in the male becomes differentiated, that is, when it is developed into an intromittent organ or "gonopod" (as designated by Geiser (1924)) already has been pointed out by Hildebrand (1917) and Mast in Barney and Anson (1921). The variation in size at which this organ may develop appears to be even greater than these writers supposed. It is evident from these data also that a considerable number of males are included among the females when all fish of about 21 mm. and over in total length are considered "adult," as the present writer did in sexing fish by the use of external characters, or as Barney and Anson (1921) did when they classed all fish of 15 mm. and over in length, to the base of the caudal, as adults. A considerable number of males, therefore, were classed as females in Table I. However, as stated elsewhere, the error in sexing is not large enough to cause the great minority of males that is shown in Table I. The greatest error in sexing, that is, the largest number of males classed as females, undoubtedly occurred during the months when the males were increasing most rapidly in proportionate numbers, as for example in September and October, for it is then that the largest number of young are maturing and many come within the "doubtful" size group. (The principal error is among fish 21 to 23 mm. in length.) Similarly, the error in sexing must be smallest when the fewest young

fish are maturing, as in July and August when the greatest disparity of the sexes occurs. The uncertainty of the sex determination from an external examination of some of the fish, classed as females in Table I., therefore, accounts for a part of the difference in sex ratio shown but probably only for a small part of it.

It is believed that ample evidence has been produced by the data in Table II. to show that a *one to one* sex ratio exists among young *Gambusia*. It would appear also from these data that this one to one ratio obtains at least until the fish become sexually mature. It is evident from Table I., however, that a "thinning out" process takes place among adult males which is very much in excess of the decrease among the adult females. A discussion of this interesting phenomenon is offered in the following section.

TABLE II.

THE SEX IN YOUNG *Gambusia*.

Size of Fish.	No. of Fish Sexed.		Remarks.
	Males.	Females.	
15 mm. and under..	239	232	
15.5 to 20 mm.....	878	853	
20 mm. and under..	181	210	This lot was not kept separate more definitely as to size in the original records.
Total.....	1,298	1,295	Very few fish of 20 mm. and under are sexually mature. These data, therefore, show the sex ratio among young *Gambusia*.
LARGER IMMATURE *Gambusia*.			
20.5 to 25 mm.....	672	988	Based on fish that were not yet sexually mature, although fish in these size groups usually have developed external sex characters.
25.5 to 30 mm.....	40	245	
Total.....	712	1,233	

A DIFFERENTIAL DEATH RATE OF THE SEXES IN *Gambusia*.

This subject is discussed at some length by Geiser (1924) who says, "The males are much less resistent to harmful environmental factors than females, and hence have a lower survival value." This author then continues to say that he has found

males killed much more readily in "high temperatures, high H-ion concentration, oxygen deficiency and concentrations of KCN." He also found a much higher death rate among males than females in *Gambusia* held in containers for shipment, as well as when confined in aquaria. Further along in the same discussion this author writes, "The males are smaller and hence are more liable to be devoured by small predaceous fish than the much larger female. Gravid female *Gambusia* in aquaria, also, attack and frequently kill the males." The present writer is in entire accord with Doctor Geiser's views and the data presented herein appear to lend support to his contentions.

The present writer in connection with his work dealing with fishes in relation to mosquito control has had occasion to make shipments of hundreds of thousand of *Gambusia*. It was noticed from time to time, that among the dead the males usually appeared to be proportionately much more numerous than the females. It, of course, was impracticable to sex fish that were being transferred from one place to another for breeding purposes, hence the percentage of deaths of each sex cannot be given for such shipments. The writer is certain, however, that at least usually the death rate among the males was higher than among the females.

In a large series of experiments performed for the purpose of determining the best method of catching and handling *Gambusia*, the adult fish were sexed and for these the results follow. The effects of high temperatures, also the effects of crowding the fish, as well as the results to be derived from the use of varying amounts of water in containers holding fish, were tested. Furthermore, an effort was made to determine, by the number of fish that survived after several days of confinement under identical conditions whether catching by seine or by dipnet was preferable.[1] The results with respect to the death rate for each set of experiments will not be given separately, as it was higher for males than for females for every condition tested. In some individual lots proportionately fewer deaths occurred among the males than among the females. Generally the reverse, however, was true, as already stated and as shown by the data that follow.

[1] The practical results derived from the experiments referred to were reported by Hildebrand (1925).

A total of 41,073 adult fish, consisting of 7,337 males and 33,736 females, were used in the experiments described in the preceding paragraph. The loss among the males was 29¾ per cent. and among the females it was 22⅓ per cent. This mortality, as a whole, was very high, that is, much higher than usually occurs in *Gambusia* that are transported from one place to another, because many of the fish, for the sake of experimentation, were held under very adverse conditions. For example, in working with the effects of high temperatures the containers were set in the sun for the purpose of determining at how high a temperature the fish could survive. The males and females, however, were subjected to the same tests and, therefore, the data should be nearly as representative of one sex as of the other. Two discrepancies, nevertheless, may be mentioned. First, in some lots all the males died before the experiments were concluded. In such cases the mortality of the females (for additional ones usually died) is greater than it would have been, if the experiments had been terminated when the last male had died. Second, it is probable that some immature males that exceeded a length of 20 mm. died and, if so, they were classed as females. Therefore, the percentage of deaths given for the females is subject to a small error, as it is somewhat higher than it should be for direct comparison with the percentage of deaths among the males. It may be concluded, then, that the differential death rate among the fish used in the experiments was greater than indicated by the data.

The data show that under artificial conditions the death rate almost constantly was higher among males than among females, and it seems probable, that the males are generally less resistant to harmful environmental factors and this probably is true in nature as Geiser (1924) has contended, as well as under artificial conditions.

The present writer has no other pertinent data to offer bearing upon the cause or causes for the greater decrease in males than females, occurring annually. Geiser (1924) argues that the smaller males are more liable to be devoured by small predaceous fish than the much larger females and he also points out that the female, herself, is an enemy of the male. Barney and Anson (1921), on the other hand, argue (see quotation, p. 393) that the

larger and more conspicuous female is the one suffering most from predatory fish. The writer believes that the former view may be correct but that in addition to fish, other natural enemies, such as water snakes, birds and insects should be considered as they all take part in destroying *Gambusia*.

Conclusion.

It has been shown quite conclusively that in immature *Gambusia* the sexes are evenly represented, as the 2,593 fish (all 20 mm. and under in length) that were examined, consisted of 1,298 males and 1,295 females. It was shown also, that a large "seasonal" variation in the abundance of males among adult *Gambusia* takes place, the data presented showing that the fluctuation in sex ratio may vary from 1 male to 2.5 females in June to 1 male to 11.3 females in August. Some evidence was produced tending to show that this great "thinning out" of males may be due to their lack of resistance to adverse environmental conditions, and the possibility that they are more extensively preyed upon by natural enemies, also, was advanced. What the greater mortality of the males may be due to, however, very largely, remains for further investigation.

Although male *Gambusia* are constantly fewer in number than females, it seems certain, nevertheless, that enough males for breeding purposes always are present. It has been shown that copulation between broods is not necessary, as females separated from males early in the spring produced young throughout the breeding season. In the spring when apparently the majority of the females are fertilized for the duration of the breeding season, the males are proportionately much more numerous. The greatest scarcity of males occurs in midsummer (July and August) when the females born the previous season have nearly all matured and quite certainly have been fertilized. At that time few males are needed. It, at least, is unquestioned that when conditions are favorable, *Gambusia* are quite capable of perpetuating their kind. Certainly few fish multiply more rapidly or become more numerous. The thinning out of males, therefore, appears to be nature's process of eliminating "surplus" animals.

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Vol. LIII *December, 1927* *No. 6*

BIOLOGICAL BULLETIN

THE RELATION BETWEEN THE RESPONSES BY *AMŒBA* TO MECHANICAL SHOCK AND TO SUDDEN ILLUMINATION.[1]

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The reactions induced in *Amœba* by mechanical shock are remarkably like those brought about by sudden illumination (Folger, '25, '26). In both cases the response consists of a cessation of movement, the animal remaining inactive for a short time, then resuming locomotion. In both cases a short period intervenes between the application of the stimulus and the response, and this period, the reaction-time, varies directly with the magnitude of the stimulation, becoming shorter as the latter increases; while the time during which the amœba is inactive, the period of quiescence, likewise dependent on the magnitude of the stimulus, becomes longer with increase of the latter. Moreover, in both instances a certain amount of time must elapse after a stimulus has been applied before the amœba will respond to a second stimulus. Thus, an exposure to light of sufficient duration to bring about a response must be followed by an absence of light or at least by a lowered intensity for a certain amount of time before the amœba will again respond to an increase in illumination. This occurrence of a period during which the amœba is apparently reverting to the condition it was in before stimulation, which I have called the period of recovery,[2] and the fact that a recovery occurs

[1] Contribution from the Zoölogical Laboratory of the University of Michigan.

[2] The term recovery has been used here simply because it is descriptive; refractory period has been employed and in the case of light dark adaptation. Recovery should not be confused with the resumption of protoplasmic flow by the amœba. The animal may have resumed locomotion and be moving

after a mechanical shock as well as after sudden illumination, is of value and has been used, as we shall see presently, for a study of the relationship which exists between the reactions caused by mechanical shock and those brought about by light.

In a study of the influence of light on *Amœba* (Folger, '25) the question occurred: since the animal must recover from the effects of a sudden illumination before it will again respond to sudden illumination, must it also recover from the effects of a mechanical shock before it will respond to sudden illumination? The answer was clearly an affirmative one. In many instances the mechanical shock entirely inhibited a response to a sudden increase in luminous intensity, when the latter was applied shortly afterward. In these instances, however, the shock was brought about by moving the coverslip with the tip of a lead pencil, a method which, while it did produce undoubted results, is extremely crude. As a better means for controlling mechanical shock has since been used, the experiment dealing with the effect of this stimulus on the response to sudden illumination has been repeated and the results are presented in the following pages.

Moreover, if a mechanical shock preceding sudden illumination influences the response to the latter, the question also arises, is the reverse true? Does sudden illumination affect the response to mechanical shock? The results of experiments designed to answer this question are also set forth in this paper.

Materials and Methods.

Specimens of *Amœba proteus* were used in the experiments, raised in small glass vessels containing a culture solution formed by adding raw hay to distilled water. Large and active individuals were selected for experimentation.

Two sets of apparati were employed, one to bring about sudden illumination, the other to cause a mechanical shock. The latter was obtained by allowing a copper wire, weighing about 300 mg., to drop through a glass tube, 68 cm. in length, which was supported by a stand and clamps in such a manner that the weight

rapidly and still not have "recovered" from the effects of stimulation as indicated by the fact that it fails to respond to a second exposure to illumination.

struck one end of the slide containing the amœba. Light was procured from a 1,000-watt, 112-volt, cylindrical Mazda stereopticon lamp, and flashed upon the amœba by means of the plane mirror of the microscope, set at an angle of 45 degrees. An intensity of about 16,000 meter candles was employed. To observe the organism when it was not illuminated by the strong light use was made of a Spencer miniature substage lamp.

The amœba to be experimented on was placed in a drop of water on a glass slide, within a ring of vaseline, and beneath a thin coverslip which was supported on one side by a small glass rod. The microscope was so arranged that when the amœba was in position under the lens, light could be flashed upon it or a mechanical shock applied at the will of the investigator, and the effect of one stimulus upon the other noted.

Experimental Results.

Table I. illustrates the effect of mechanical shock upon the response to light. The experiment from which the data in this table were derived consisted of a number of tests, in each of which the amœba was exposed to strong illumination, and in every other one of which it was subjected to a mechanical shock before being illuminated. This shock was of sufficient magnitude to cause a cessation of movement, and the animal was exposed to light immediately on the resumption of flow. An interval of three minutes between tests permitted a recovery from the effects of previous stimulation. As shown in the table the amœba failed to respond to light 4 times out of 6 trials when a mechanical shock

Table I.

Illustrating the Effect of Mechanical Shock upon the Response to Sudden Illumination.

In each trial the amœba was exposed to strong illumination, and in every other one it was subjected to a mechanical shock before being illuminated. Three minutes were allowed between tests.

	Number of Trials.	No Reactions.
Sudden illumination alone	5	0
Sudden illumination following shortly after a mechanical shock	6	4

preceded the exposure to illumination, while it responded 5 times out of 5 trials when previous stimulation by mechanical shock was lacking. These results are entirely in accord with previous observations and leave no doubt that mechanical shock does affect the response to light.

Table II. is the record of an experiment in which the procedure

TABLE II.

SHOWING THE EFFECT OF SUDDEN ILLUMINATION UPON THE RESPONSE TO MECHANICAL SHOCK.

In each trial the animal was subjected to a mechanical shock, and in every other one it was illuminated before being exposed to shock. Three minutes were allowed between tests.

Individual No.		Number of Trials.	No Reactions.
1	Mechanical shock alone	5	0
	Mechanical shock following immediately after sudden illumination	6	3
2	Mechanical shock alone	6	1
	Mechanical shock following immediately after sudden illumination	8	4
3	Mechanical shock alone	6	1
	Mechanical shock following immediately after sudden illumination	6	5
4	Mechanical shock alone	4	0
	Mechanical shock following immediately after sudden illumination	6	4
Totals	Mechanical shock alone	21	2
	Mechanical shock following immediately after sudden illumination	26	16

in the experiment just described was reversed. Here tests in which the animal was stimulated by illumination and immediately on the resumption of movement given a mechanical shock alternated with tests in which a mechanical shock alone was used as the stimulating agent. As shown in the table, individual No. 1 reacted 5 times out of 5 trials when stimulated by mechanical shock alone, while it reacted to the same stimulus only 3 times out of 6 trials when the mechanical shock was preceded by ex-

posure to light. Individual No. 2 reacted 5 times out of 6 trials when subjected to mechanical shock alone, and only 4 times out of 8 trials when the mechanical shock was preceded by illumination. Altogether, the 4 animals used in the experiment responded to mechanical shock 19 times out of 21 trials when this stimulus did not follow an exposure to light, while they failed to respond to it 16 times out of 26 trials when it did follow illumination, thus indicating that exposure to light does influence the response to mechanical shock. Even more convincing, however, are the results of an experiment to be described in the next paragraph.

It has already been shown (Folger, '26) that the length of time that an amœba remains inactive after stimulation by a mechanical shock is markedly affected by the length of time that has elapsed since a previous mechanical shock, and that, within limits, this quiescent period increases with increase in the length of time since the previous stimulation. Thus, the period of quiescence resulting from a mechanical shock which follows 30 seconds after a preceding similar shock is not likely to be nearly so long as that brought about by a shock following after an interval of 60 seconds. Table III. records an experiment in which somewhat similar results were obtained, but in which the organism was first stimulated by light and then by mechanical shock. From the table it is seen that the amœba reacted only twice out of 4 trials when 15 seconds were allowed for recovery between stimulation by light and by

TABLE III.

SHOWING THE EFFECT OF LIGHT ON MECHANICAL SHOCK.

In each test the amœba was first exposed to light and then allowed to recover from the effects of this stimulus for the time indicated in the table, after which it was subjected to a mechanical shock. The period of quiescence, recorded in the last column, consists of the time during which the amœba was inactive after it had been subjected to a mechanical shock.

Time Allowed for Recovery Between Sudden Illumination and Mechanical Shock (Sec.).	Reactions.	No Reactions.	Average Period of Quiescence (Sec.).
15	2	2	4.5
20	3	0	5.8
25	4	0	9.1
35	2	0	20.0

mechanical shock, and that the period of quiescence amounted to 4.5 seconds. Twenty seconds for recovery resulted in 3 reactions out of 3 trials, with an average period of quiescence of 5.8 seconds, 25 seconds for recovery resulted in 4 reactions out of 4 trials, with an average period of quiescence of 9.1 seconds, and 35 seconds for recovery resulted in 2 reactions out of 2 trials, with an average period of quiescence of 20 seconds.

Thus it appears that not only does sudden illumination affect the response of an amœba to mechanical shock, but that it has precisely the same effect as another mechanical shock.

Discussion.

A similarity of response to various kinds of stimuli has been noted in other organisms. Ewart ('03) has collected considerable data concerning protoplasmic streaming in plant cells, especially in the cells of *Chara* and *Nitella*. He and others have found that the cells react in a very characteristic way to various stimulating agents, the response consisting, just as in *Amœba,* of a temporary cessation of movement. The best quantitative results were obtained by means of a mechanical shock, brought about by dropping a weight on the coverslip beneath which the streaming cells had been placed. By using weights of various sizes a gradation in the magnitude of the shock was possible. Ewart discovered that streaming did not stop immediately on application of the stimulus, but after the intervention of a reaction-time, which was longer after a light shock than after one of greater magnitude and which in the event of a sub-minimal stimulus might amount to 7 or 8 seconds. He found, furthermore, that the time during which movement remained suspended likewise depended on the magnitude of the shock, streaming recommencing much sooner after a slight shock than after a heavy one. Various other stimulating agents, including light, heat, electricity, and change in concentration of the surrounding medium, gave very similar results, although in no instance was so accurate a quantitative measurement obtained as with mechanical shock. Ewart also noted that the application of one kind of stimulus may tend to inhibit the response to another kind.

The refractory period used as a basis for the experiments presented in this paper, during which recovery from the effects of previous stimulation occurs, has long been known, especially when light has been used as the stimulating agent. In this case the term dark adaptation has been used. An animal must be dark-adapted before it will respond to illumination. This necessity for dark-adaptation has led to an explanation of the response to light which involves the presence in the organism of a substance capable of a reversible photochemical reaction (Mast, '07; Hecht, '18). The reaction is thought of as being initiated by the conversion of a photosensitive substance into its precursors, and dark-adaptation as the reforming of this substance from the precursors.

As we have just seen, however, recovery from the effects of a mechanical shock may be necessary before a response to sudden illumination is possible, and it would therefore seem that the same processes are involved in the refractory periods which follow the two types of stimuli. If so, since a photochemical reaction cannot occur in the case of mechanical shock, it is evident that in *Amœba,* at least, dark-adaptation must consist of something besides the reforming of a photosensitive substance from its precursors.

Summary.

1. *Amœba* responds both to mechanical shock and to sudden illumination by a cessation of movement, which does not take place immediately on stimulation, but after a considerable reaction-time.

2. In both cases the reaction-time depends upon the magnitude of the stimulus, becoming longer as the intensity of the stimulating agent increases.

3. In both instances the time during which the amœba is inactive also depends upon the magnitude of the stimulus, becoming longer as the latter increases.

4. After an amœba has been exposed to light it is necessary that a certain interval of time elapse before it will again respond to sudden illumination. Likewise, after a mechanical shock the amœba must be allowed time for recovery before it will respond to a second shock.

5. After a response to light time must be allowed for recovery before the amœba will react to mechanical shock and vice versa.

6. The effect of one kind of stimulus upon a response to another kind leads one to infer that the processes occurring during the refractory periods following the reactions caused by mechanical shock and by sudden illumination are basically the same.

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CHROMOSOME NUMBERS IN THE GENUS *BURSA*.

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In a recent paper, Shull divides the genus *Bursa* into two groups on the basis of genetical studies. The *Bursa-pastoris* group includes six species and two subspecies, as follows: *Bursa bursa-pastoris* (L.) Britton, *B. bursa-pastoris apetala* (Opiz), *B. Heegeri* (Solms-Laubach), *B. occidentalis* Shull ined., *B. occidentalis madeiræ* Shull ined., *B. orientalis* Shull ined., *B. djurdjuræ* Shull ined., and *B. penarthæ* Shull ined. The *Rubella* group includes four species, as follows: *B. grandiflora* (Bois.), *B. rubella* (Reut.), *B. Viguieri* (Blaringhem), and *B. tuscaloosæ* Shull ined.

Crosses between members of the same group yield more or less fertile F_1 hybrids. Crosses between any species of one group and any species of the other group yield sterile hybrids in the F_1. Duplication of factors for certain characters is found in the *Bursa-pastoris* group of species, but not in the *Rubella* group.

Rosenberg (1904) had reported the haploid number of chromosomes of *Bursa bursa-pastoris* to be 16, and the diploid number to be 32. These counts were confirmed by Laibach (1907). Marchal (1920) reported 16 haploid chromosomes for *Bursa Heegeri*, and 8 haploid chromosomes for *Bursa Viguieri*. Knowing that two members of the *Bursa-pastoris* group possessed 16 haploid chromosomes, and that one member of the *Rubella* group possessed 8 haploid chromosomes, Shull suspected that the cause for the inter-sterility of these two groups might lie in a difference in chromosome number between the two groups. Since rather unusual opportunities were offered for securing material from Professor Shull's pedigree cultures of *Bursa*, I undertook a cytological examination of the various species of the two groups.

The cells selected for study were the pollen mother cells, since at the maturation divisions they offer relatively large cells with the

[1] This investigation was carried out principally in the cytological laboratories of Princeton University.

reduced number of chromosomes, and can always be recognized. The material was fixed in Carnoy's acetic-alcohol-chloroform, and stained with Heidenhain's iron hematoxylin. No counterstain was used. The maturation divisions apparently consume very little time, for metaphase stages were never found in material which was fixed later than five minutes after collection. Since the buds are very small, dissecting out the anthers in this limited time was impracticable, so all of the unopened buds of a raceme were fixed at once. The raceme was then sectioned, usually at 6 micra. The cells at the time of the first maturation division are about 10 micra in diameter. It was possible to find cells cut at right angles to the spindle, so that the metaphase chromosomes were disposed in a horizontal plane. Counts were made in all of the species of the *Rubella* group. Marchal's count of 8 haploid chromosomes for *Bursa Viguieri* was confirmed, and 8 haploid chromosomes were found in the other three species. In *B. grandiflora* the diploid count also was made, and found to be 16, but somatic counts were not made for the other species of this group. In the *Bursa-pastoris* group not all of the species were counted, as several were unavailable, and in one available species no count was made. Rosenberg's count of 16 haploid chromosomes, 32 diploid chromosomes for *B. bursa-pastoris* was confirmed, and 16 haploid chromosomes found for *B. occidentalis, B. orientalis,* and *B. bursa-pastoris apetala.* As far as counted, these two groups are as sharply divided on the basis of chromosome numbers as on the results of breeding experiments.

We may tabulate the chromosome numbers in the genus *Bursa* as follows:

	Haploid.	Diploid.	Author.
B. bursa-pastoris (L.) Britton	16	32	Rosenberg (1904), Laibach (1907), Author (1927)
B. Heegeri (Solms-Laubach)	16		Marchal (1920)
B. occidentalis Shull ined.	16		Author (1927)
B. Orientalis Shull ined.	16		Author (1927)
B. bursa-pastoris apetala (Opiz)	16		Author (1927)
B. grandiflora (Bois.)	8	16	Author (1927)
B. rubella (Reut.)	8		Author (1927)
B. Viguieri (Blaringhem)	8		Marchal (1920), Author (1927)
B. tuscaloosæ Shull ined.	8		Author (1927)

It is planned to continue this work next year when more material is available, completing the counts for the species of the *Bursa-pastoris* group, and examining a certain number of the inter-group hybrids. I had at first planned to study the details of the maturation mitoses, but the small size of the chromosomes and the difficulty of securing satisfactory material in the right stages of division make it unlikely that this will be attempted.

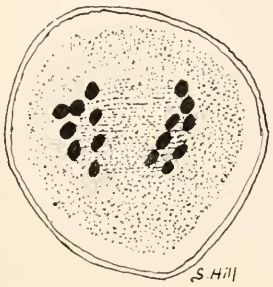

FIG. 1.

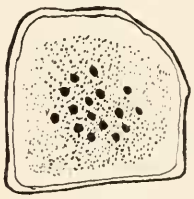

FIG. 2.

FIG. 1. Anaphase of first maturation division of *B. grandiflora*. × 3,000.
FIG. 2. Metaphase of first maturation division of *B. occidentalis*. × 3,000.

Figures are given for *B. grandiflora* and *B. occidentalis* only, but in the later paper figures will be given for all of the species counted.

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ULTRAVIOLET RADIATION AND THE FERTILIZATION REACTION IN *ARBACIA PUNCTULATA*.

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For a general consideration of the fertilization problem, the reader is referred to Chapter VIII., "Fertilization," by F. R. Lillie and E. E. Just, in Cowdry's "General Cytology," 1924; also to "Problems of Fertilization," by F. R. Lillie, 1919. In this paper, the studies of individual authors will be cited only where it seems desirable to refer to results which have a particular bearing on those obtained in the experiments about to be described.

The fertilization reaction may be modified by the direct action of a physical or chemical agent upon either or both of the sex components, or upon the zygote. Such interference results in the production of differentially modified larvæ, of irregular cleavage (often incomplete), of lack of membrane formation, or even of cytolysis. The magnitude of the injury is a function of the dosage and the time at which the exposure is made. This is easily demonstrable with *Arbacia* and ultraviolet radiation (Hinrichs, '26, *a*, *b*).

Furthermore, it is possible by means of radiation to interfere with the characteristic behavior of the sex cells, *e.g.*, the motility of the sperm is lost (Lillie and Baskervill, '22, and Hinrichs, '26*c*), and the agglutinability by normal egg-water is reduced or lost. The permeability of both sex components is altered, and the fertilizin-producing capacity of the egg is lost, following radiation.

Methods.—In the experiments involving the radiation of eggs, sperm, or egg-water, the material was placed in open dishes (immersed in a water bath) at a distance of 12.5 cm. from the lamp. (A few exposures were made at 23.0 cm. from the lamp, for comparison.) The radiation source was a Cooper-Hewitt quartz mercury-vapor lamp, operating at 110 volts D.C. and about 4 amperes.

Variations in technique will be noted as the various groups of experiments are described. The following topics are considered; radiation of unfertilized eggs, "egg-water," sperm suspensions, and agglutinated sperm; adsorption of the colloidal components of egg-water, and their partial recovery by HCl.

A. Experiments with Unfertilized Eggs.—Unfertilized eggs were radiated "dry," for varying periods of time (15 sec. to 20 min.) and tested for fertilizin production, as follows; equal volumes of sea-water (5–10 cc.) were added to equal quantities of eggs immediately after radiation and allowed to stand for from 15 min. to 2 hr. The water was then decanted off, and its agglutinating power measured. This was repeated from 1 to 5 times. The agglutinating power of a given sample of egg-water was measured by successively diluting the sample until it no longer gave a characteristic normal agglutinating reaction with a 1 per cent. sperm suspension. The last effective dilution is an

TABLE I.

ULTRAVIOLET RADIATION AND FERTILIZIN PRODUCTION.

Date.	Time after Exp. (Min.)	Length of Period of Radiation. (In min.)									
		0	¼	½	¾	1	3	5	10	15	20
		A. U.									
8/26	30	6.0						15	30	15	
	60	1.5						—	1.5	—	
27	30	70				110	90	70	70		
	60	3				18		6	6		
28	15	60						30			
	30	7						7			
	45	—						—			
28	15	350				700			55		30
	30	60				60			6		1.5
	45	60				60			6		—
	60	60				30			6		—
30	15	600	600	600	1,200						
	30	"	"	"	"						
	45	"	"	"	"						
	60	"	"	"	"						
	240	1,200	2,400	2,400	4,800						

A. U.—Agglutinating units. Tests were made against a 1 per cent. sperm suspension.

expression of the agglutinating power in terms of "agglutinating units." This is the method used by Lillie in determining the agglutinating strength of a given sample of egg-water (F. R. Lillie and E. E. Just, '24, p. 487). A summary of a number of such experiments appears in Table I.

It will be seen that the rate of fertilizin production is at first increased, then decreased. In 3 experiments, the increase came with exposures of 1 minute or less. In the other two experiments, all exposures exceeded 5 min., and in one case the maximum increase came at 10 min. Successive tests for agglutinating power showed a rapid decrease in rate of fertilizin production, both in radiated and in non-radiated lots of eggs.

Samples of eggs fertilized with normal sperm at the time of testing for agglutinating power indicate a falling off in fertilizability and general viability running parallel to the decrease in rate of fertilizin production.

TABLE II.

FERTILIZABILITY OF RADIATED EGGS.

Min. Exp.	Min. after Exp.	A. U.	Stage of Development Reached. (In per cent.)			
			Morula.	Irregular Cleav.	Membranes Only.	Cytolysis.
0	15	350	98	1	1	—
	30	60	99	1	—	—
	45	60	97	2	1	—
	60	60	96	2	2	—
1	15	700	88	10	2	—
	30	60	50	45	5	—
	45	60	55	35	10	—
	60	30	35	53	12	—
10	15	55	68	5	3	24
	30	6	30	4	2	64
	45	6	25	10	5	60
	60	6	25	3	8	64
20	15	30	—	10	5	85
	30	1.5	—	3	5	92
	45	—	—	3	3	94
	60	—	3	5	3	89

A. U.—Agglutinating units.

The percentage of normal development is reduced by radiation (Hinrichs, '26*b*), and also by a delay in fertilization (F. R. Lillie,

'15). In these experiments, cleavage is irregular and 50 per cent. of the eggs are cytolyzed following a 3 min. exposure, and from 50–75 per cent. are cytolyzed by exposures of 5 min. or longer. In general, where the percentage of abnormal cleavage and subsequent abnormal development is high, the fertilizin production is correspondingly low. Lillie and Just, '24, p. 487, call attention to the coincidence of the production of agglutinating substance by mature eggs with the fertilizable period of such eggs. (Lillie in 1914, and Just, in 1919, determined such a relation for *Arbacia* and *Echinarachnius,* respectively.)

The production of fertilizin is also decreased as membranes begin to form. A series of acid-treated eggs (with the jelly removed by the acid) showed no appreciable difference in the amount of fertilizin produced during successive intervals when compared with untreated eggs. (See discussion, Lillie and Just, '24, p. 492.) These tests were made to determine whether fertilizin was retained by the jelly and then given off into the seawater in large amounts as though secreted by the jelly. The jelly appears to adsorb fertilizin, as will be described later, and the adsorbed fertilizin retains enough of its activity to attract and agglutinate sperm to the egg surface. Lillie suggests that the jelly acts as a protection against loss of fertilizin (Lillie, '19, p. 142). Sperm show a greater avidity for eggs radiated for short intervals than for non-radiated eggs, a fact suggesting an increased production of some substance which attracts them. (See also Table I.)

It will be seen that the rate of production of fertilizin in radiated eggs decreases as the time following radiation increases, and at a more rapid rate than in non-radiated eggs. The total production in radiated lots is also less than in normal eggs. This is probably due to an effect of ultraviolet radiation upon the egg cortex, which is supposed to be the seat of fertilizin production.

B. Radiation of Egg-water.—Egg-water contains several complex substances given off by the eggs. (See studies of Lillie and Just, '24, p. 483.) When such egg-water is exposed to ultraviolet radiation, its various components appear to be separately affected. The echinochrome pigment is faded, and the activating and agglutinating substances lose their effectiveness, all at different

rates. Colorimeter measurements were made of the rate of fading of echinochrome. The results of such experiments are recorded in Table III.

TABLE III.

THE FADING OF ECHINOCHROME PIGMENT BY ULTRAVIOLET RADIATION.

Distance from Lamp.	Length of Period of Exposure. (In sec.)											
	0	2	4	5	6	8	10	15	20	25	30	35
12.5 cm.....	*100%	100	94		72	62	50	52	49			
23.0 cm.....	100%			81			67	51	49	46	35	35

* Results are given as per cent. of color remaining after each exposure. Non-radiated egg-water is used as the control.

Non-radiated egg-water was placed in one tube of the instrument and used as a color standard. Radiated samples were placed in the comparator tube. Three readings were taken for each sample, and the ratios of color intensities calculated. In each case, an average of the three results was compared with that of the non-radiated control. The final ratios appear in Table III. It will be seen that the ratios of color intensity in radiated lots as compared with color intensities in non-radiated lots decrease more rapidly as the length of the period of radiation increases.

Normal egg-water, when added to a normal sperm suspension, produces activation and agglutination of sperm, *i.e.*, the spermatozoa form regular spherical masses which persist for a short period of time, and then break up. The persistence of the masses is a function of the condition of the sperm, and of the concentration of active fertilizin in the egg-water. Radiation of egg-water causes a reduction and finally a loss of agglutinating power. Fewer dilutions of a given sample of egg-water are possible before the last effective dilution is reached. The results of radiating egg-water and the consequent loss of agglutinating power will be found in Table IV.

The rate of loss of agglutinating power does not exactly parallel that of the loss of color. It appears that ultraviolet radiation is more effective in accelerating the loss of agglutinating power than the loss of color. A 2 min. radiation at 12.5 cm. reduces the agglutinating power of a sample of egg-water by 50 per cent., but

only a 6 per cent. reduction of color loss appears during the same period. In this case, 68 cc. of dry eggs were covered with enough sea-water in a graduated cylinder to reach the 250 cc. mark. The suspension was allowed to stand for 3 hr. before the egg-water was decanted off. This egg-water was then exposed to radiation for varying periods of time, and its agglutinating power tested against a 1 per cent. sperm suspension. In each case, the final agglutinating dilution was noted. (See Table IV.)

TABLE IV.

THE EFFECT OF RADIATION ON THE AGGLUTINATING POWER OF EGG-WATER.

Distance from Lamp.	Length of Period of Exposure. (In sec.)											
	0	2	4	5	6	8	10	15	20	25	30	35
	Agglutinating Units Remaining.											
12.5 cm. .	1,200	600	600		300	150	150		<1			
23.0 cm. .	1,500			900			850	300	150	88	63	18
	Agglutinating Units Lost. (In per cent.)											
12.5 cm. .		50	50		75	88	88		99+			
23.0 " .				40			44	80	99	94	96	99+
	Per Cent. Color Lost. (See Table III.)											
12.5 cm. .		0	6		28	38	50		51			
23.0 cm. .				9			33	49	51	54	65	65

Prolonged radiation of egg-water prevents the activation and agglutination of sperm, and may even produce a lethal effect on sperm. When radiated egg-water is added to normally agglutinated sperm, the clusters become " loose " and permanent, and the sperm inactive. Sperm inactivated by radiated egg-water may be reactivated by normal egg-water, so that normal but small, reversible clusters may form.

The agglutinating power of a given sample of egg-water may have been entirely lost following radiation, yet the same sample may still be capable of stimulating sperm to greater activity. Either the sperm-stimulating and sperm-agglutinating substances are not identical (Lillie and Just, '24, p. 483) or a lesser amount

of the substance is necessary to activate than to agglutinate sperm. The former alternative is probably correct. (See also Glaser, '21, Woodward, '18, and Clowes and Bachman, '20.)

Temperature, in combination with the effects of radiation, is also a factor in determining the loss of agglutinating power and of color. To illustrate: samples of egg-water were radiated at 38°, 21°, and 4° C. Table V. records the results of such experiments. Only percentages of loss are given.

TABLE V.

EFFECT OF RADIATION AT VARIOUS TEMPERATURES.

Distance from Lamp.	Percentage Loss.							
	A. Agglutinating Units.				*B*. Color.			
	Min. rad.	Temperature			Min. rad.	Temperature		
		38° C.	21° C.	4° C.		38° C.	21° C.	4° C.
12.5 cm.....	5	92	50	75	5	33	29	16
	10	100	90	88	10	64	52	50
	20	100	100	100	20	63	68	59
	Min. rad.	Temperature.						
		38.5° C	22.5° C	4° C				
23.0 cm.....	5	50	50	88				
	10	99	90	99				
	20	99+	99	100				

At any given temperature, the rate of loss of color is slower than that of agglutinating power; also, the rate of color loss is greater at higher than at lower temperatures, while the agglutinating power is lost more quickly at both high and low temperatures than at sea water temperature.

Normally the fertilizin content of egg-water is fairly stable, particularly if the egg-water is covered with a toluol film to minimize bacterial infection. (See also Lillie and Just, '24.) The greatest falling off in agglutinating power of such a sample comes during the first day or two, after which the agglutinating power remains fairly constant. (See Fig. 1.)

The radiation of egg-water produces no significant change in pH in either direction, although in some experiments a slight in-

crease in acidity was noted. Following adsorption of the colloid components of egg-water by charcoal, an increase in alkalinity occurred.

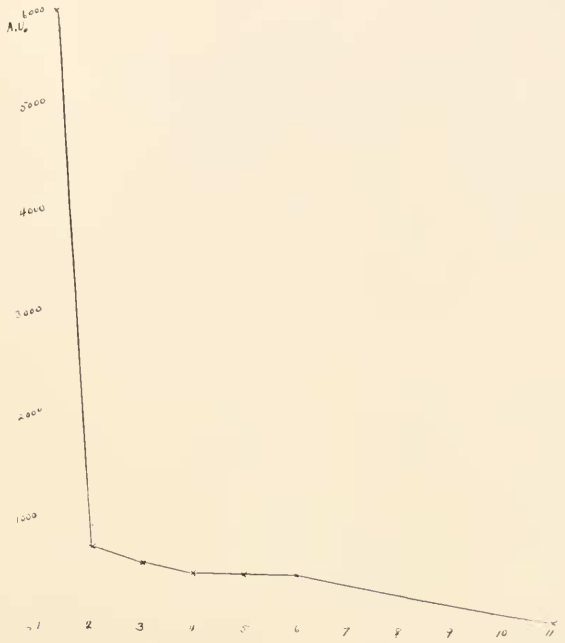

FIG. 1. Normal decline of agglutinating power of a sample of non-radiated egg-water. Ordinates, agglutinating units. Abscissæ, duration of experiment, in days.

Charcoal Adsorption.—Equal weights of animal charcoal, previously washed and dried, were added to equal quantities of a series of dilutions of egg-water whose agglutinating power was known. The charcoal egg-water mixture was shaken and then filtered through filter-paper, and its agglutinating power again tested against the same sperm suspension. The filtrate was colorless, and had lost from 90 to 100 per cent. of its agglutinating power, indicating a high adsorption coefficient. (See Table VI.) Both echinochrome and fertilizin had been adsorbed. (In this connection see also Glaser, '21*a*.)

It will be seen that relatively more fertilizin is adsorbed from samples of egg-water which have been diluted. The data for the

experiment of 8/13 (Table VI.), are represented in Fig. 2. The agglutinating units for each dilution, before and after adsorption by charcoal, are indicated.

TABLE VI.

ADSORPTION OF FERTILIZIN BY CHARCOAL.
(Per cent. agglutinating units adsorbed.)

Date.	*A.U. —0.	A.U. —.1.	Conc. Sperm	Charcoal gm./cc.	Dilution of Egg-water.										
					0	.9	.8	.7	.6	.5	.4	.3	.2	.1	.05
8/12	1,125	5	4%	2 gm. / 100 cc.	95†	93	100	100	100	100	100	100	100	100	
13	384	—	1%	1 gm / 100 cc.	93	96	96	95	95	98	98	99+	100	100	100
14	384	.8	1%	1.5 gm / 100 cc.	43	86	84	91	89	88	84	95	99	99	
17	315	15	1%	200 mg. / 100 cc.	30	40	40	32	31	81	76	81	76	53	
18	192	1.5	1%	1 gm. / 25 cc.	54	44	68	64	58	88	84	89	92	89	
19	3,000	—	1%	1 gm. / 25 cc.	95	96	96	95	96	99	99+	99+	100	100	

* A. U.—0 number of agglutinating units at the start. (No dilution.)
A. U.—.1 number of agglutinating units at a dilution of 1 part egg-water to nine parts sea-water.
† Figures represent per cent. agglutinating units adsorbed.

Further evidence for the high adsorption constant of fertilizin is obtained as follows: when a suspension of fresh sperm is added to charcoal, the sperm swim actively about, often coming into contact with the bits of charcoal, but not remaining attached to them. If a few drops of egg-water are now added to this mixture, no agglutinated masses are formed in the spaces between the pieces of charcoal, but the fertilizin seems to be immediately adsorbed by the charcoal, and the sperm are attracted by it, and become agglutinated to each other in an irregular film around the bits of charcoal, and remain there. The masses are irreversible and permanent, due probably to the high concentration of still active fertilizin about the charcoal, and the lack of sufficient activating

substance to stimulate the sperm to activity. The activating component of egg-water appears not to be adsorbed by charcoal, or less so than the agglutinating component.

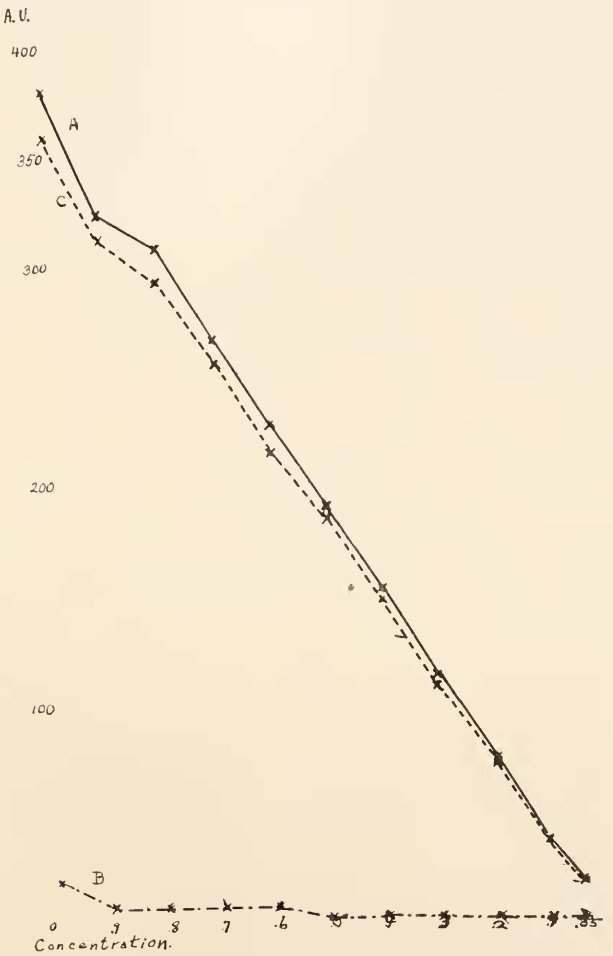

FIG. 2. Adsorption of fertilizin on charcoal. Ordinates, agglutinating units. Abscissæ, concentration of egg-water used. A. U., agglutinating units. *A*, agglutinating units in sample before adsorption. *B*, agglutinating units in sample after adsorption. *C*, agglutinating units adsorbed.

When a few drops of concentrated sperm are added to egg-water containing a small amount of charcoal, the sperm immediately attack the bits of charcoal and rotate them about much as

they do ripe eggs. The retention of considerable activity by adsorbed fertilizin suggests its enzyme-like nature. Such behavior has been noted for pepsin by Kikawa, '23. Richards and Woodward, '15, and Lillie, '21, have ascribed an enzyme-like nature to fertilizin.

When ripe unfertilized eggs are added to a charcoal-fertilizin system, and then fresh sperm are added, the sperm show a preference for the eggs, rather than for the bits of charcoal. The concentration of active fertilizin is probably greater about the egg-surface due to continued production of fresh fertilizin by the egg.

It was possible to recover a small amount of the adsorbed fertilizin by washing the charcoal with slightly acidified sea water. (See Table VII.) Glaser, '21*b*, reports the recovery of some fertilizin by HCl. Neither iso-amyl alcohol nor saponin were effective in displacing the fertilizin from adsorption.

Table VII.

The Recovery of Fertilizin Adsorbed on Charcoal.
($\frac{N}{10}$ HCl was used.)

Before Adsorption.		After Adsorption		% ads.	pH of Wasl Water.	A.U. after Washing.	% Recovered.
pH.	A.U.	pH.	A.U.				
7.20	160–320	7.70	20–40	87.5	3.8	1 —32	11.4
7.45	3,500	8.0	150	96	6.8	—	—
8.00	1,250	8.1	300	76	6.7	3	1.
7.45	3,500	8.0	150	96	6.7	12	8.
7.65	1,500	7.8	1.5	99.9	7.1	—	—
7.80	150	7.8	—	100.	6.6	—	—
7.35	1,500	7.8	6	99.6	8.1	—	—
6.60	300	6.6	12	96.	8.2	—	—

When a charcoal-fertilizin system is washed with sea-water, the filtrate will be found to activate but not to agglutinate sperm. Apparently the activating substance is not adsorbed to the same degree as are the other substances in egg-water.

There seems to be an optimum pH range for the adsorption for fertilizin by charcoal, as well as for its effectiveness as a sperm-agglutinating agent. This optimum pH range, like that found by Clowes and Smith for fertilization, hovers around neutrality. (See Clowes and Smith, '22, '23, Smith and Clowes, '24, *a*, *b*, *c*.)

Measurements of pH in these experiments on fertilizin were made by the use of indicators. In the region of 6.8 to 8.2, from 75–87.5 per cent. of the fertilizin was adsorbed. In another series, at 7.5 to 7.9, the percentage of adsorption was from 96–100.[1] (See Table VIII.)

TABLE VIII.

EFFECT OF pH ON ADSORPTION.

Before Adsorption.		After Adsorption.		Per Cent. Agglutinating Units Adsorbed.
pH	A.U.	pH	A.U.	
9.8	120	9.8	120	—
9.0	120	9.0	60	50
8.2	120	8.2	30	75
7.5	120	7.8	15	87.5
7.45	240	7.8	30	87.5
7.2	240	7.7	30	87.5
7.1	240	7.5	30	87.5
7.0	120	7.45	15	87.5
6.8	60	7.2	15	75
5.4	30	6.0	15	50
4.8	30	6.0	30	—
3.6	6	4.4	—	—

Radiation of Sperm Suspensions.—Earlier work with *Arbacia* sperm has shown that a loss of motility and a reduction of fertilizing power follow the exposure of sperm suspensions to ultraviolet radiation. (Lillie and Baskervill, '22, Hinrichs, '26*c*.) In these earlier experiments, it was also noted that radiated suspensions of sperm had lost some of their opalescence and appeared cleared in transmitted light. This was probably due to the settling out of suspension of irregular aggregations of sperm; such clumping or agglutination results from exposure to ultraviolet radiation. (Hinrichs, '26*c*.) In the present series of experiments with sperm, it was noted that the decrease in opacity to light was proportional to the dosage of radiation a given sperm suspension had received. The results are recorded in Table IX.

The relative opacity was measured by means of a nephelometer. A 1 per cent. sperm suspension was used as stock, and from 1 to 20 drops thereof per 100 cc. of sea-water were exposed to radiation at 12.5 cm. from the lamp, for periods of from 15 sec. to 15 min. As in previously reported experiments, the more dilute sus-

[1] More work is planned on this phase of the problem.

pensions were more susceptible to changes induced by radiation, and the effect was graded by exposure.

TABLE IX.

LOSS OF OPALESCENCE OF SPERM SUSPENSIONS FOLLOWING RADIATION.

Time Exp. in Sec.	Concentration of Sperm. (Drops of 1 Per Cent. Suspension per 100 cc. Sea-water.)					
	20	15	10	5	3	1
15	*1.12	.81	.90	.97	1.19	.90
30	1.04	.84	.95	1.15	1.08	1.02
60	1.17	.87	.75	.97	1.33	1.00
180	1.11	.85	.94	1.05	.81	.83
300	1.01	.84	.77	.82	.65	.59
600	.85	.65	.68	.51	.61	.32
900	.69	.57	.46	.53	.41	.24

* Figures represent relative opalescence of radiated sperm compared with non-radiated sperm.

There are characteristic differences between normally agglutinated masses of sperm, and those induced by ultraviolet radiation. The latter are irregular and permanent, and the individual sperm inactive. The whole clump, in each case, appears to have been formed by sperm which had become agglutinated to each other, and had almost immediately lost their motility, remaining applied to each other by sticky surfaces. Such masses resemble the ones described by various other investigators and are generally known to be toxic and irreversible. (For discussion, see Lillie and Just, '24, p. 489.)

When masses of sperm, which had been normally agglutinated by egg-water, are radiated, several things may happen, according to the intensity and duration of the radiation, and the time of its application with respect to the "age" of the agglutinated mass. When freshly agglutinated sperm are but slightly radiated, the clump breaks up almost immediately. If a slightly longer exposure is given, the sperm appear to be paralyzed and the mass becomes permanent, and more or less irregular in shape.

When sperm are slightly radiated, and then treated with normal egg-water, the onset of agglutination is delayed. Motility is greatly decreased. Lillie, '12, and Loeb, '14, found the rapidity

of the onset of agglutination to be a function of sperm activity. The time during which the agglutinated masses remain intact also decreases with increased dosage. (See Fig. 3.)

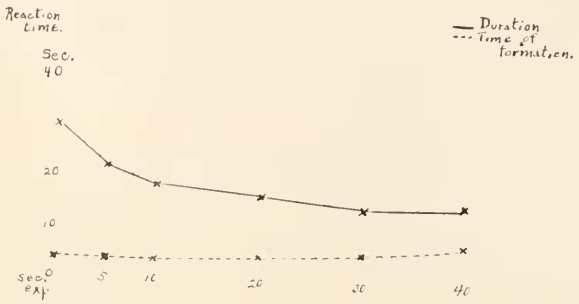

FIG. 3. Delay in agglutinating reaction following radiation of sperm. Ordinates, number of seconds elapsed before the onset of agglutination with normal egg-water. Abscissæ, length of period of exposure, in seconds.

The same conditions are found when egg-water is radiated for a short interval, and then used to agglutinate normal sperm. Its agglutinating capacity is reduced. Agglutination is delayed, and the masses persist for a shorter period of time than when normal egg-water is used with normal sperm. Longer exposures of either egg-water or sperm, previous to mixture with normal sperm or egg-water, respectively, produce larger irregular permanent masses which "run into each other" forming a kind of reticular mass throughout the suspension.

TABLE X.

LOSS OF AGGLUTINABILITY OF SPERM SUSPENSIONS FOLLOWING RADIATION.

A.		*B.*			
Time Exp. in Sec.	Greatest Effective Dilution.	Conc. Sperm in Per Cent.	Greatest Effective Dilution.		% Loss.
			Non-rad.	Rad.	
0	4,000–8,000	2.5	8,000–16,000	8,000–16,000	—
15	2,000–4,000	2.0	4,000–8,000	2,000–4,000	50
30	200–300	1.5	500–1,000	40–80	92
60	20–40	1.0	100–200	0–10*abn.	96.7
120	—				

A. Sperm in concentration of 3 drops of 1 per cent. suspension per 10 cc. sea-water.

B. Sperm in various concentrations radiated for 30 sec. each. * *abn*- aggregates were abnormal in appearance, and were permanent and "stringy."

Since the normal agglutinability of sperm suspensions is reduced by ultraviolet radiation, a stronger concentration of egg-water is necessary to produce agglutination in radiated sperm. Table X records the results of such an experiment. While the control sperm may be agglutinated with egg-water diluted about 6,000 times, sperm radiated for 60 sec. will agglutinate with egg-water diluted only about 30 times. Radiation for 120 sec. reduces agglutination to zero.

Radiated sperm suspensions show no change in pH detectable by the indicator method. Higher concentrations of sperm show a slight increase in relative viscosity, as measured by a stalagmometer. This bears out the suggestion made above regarding the increased adhesiveness of sperm heads in aggregations appearing in radiated suspensions. Apparently ultraviolet radiation changes the consistency and permeability of the sperm surface causing the sperm to become more "sticky." Sampson, '26, describes a similar change in the consistency of sperm agglutinated by normal egg-water. Glaser, '14*b*, found that egg extracts increased the permeability of cells. Other workers have reported similar results.

There appears to be a decrease in surface tension following the radiation of sperm suspensions. The relative permanence of foam structure formed the basis, for the above suggestion, following Bartsch, '26. 25 cc. of each of the various sperm suspensions were shaken in small bottles until frothy. Examinations were made after 30 and 90 minutes. It will be seen from Table XI. that the stronger suspensions of sperm had more froth than the more dilute suspensions, and that in both strong and dilute suspensions, the number of bubbles was increased by radiation, as indicated by the number of ×'s in the table.

General Discussion.—From the fact that the degree of viability and fertilizability of *Arbacia* eggs show a parallelism with the power to produce fertilizin, it may be argued that fertilizin plays an essential rôle in the fertilization reaction. (See Lillie and Just, '24, p. 486.) There appear to be two distinct substances yielded by the egg to the sea-water, one of which stimulates the sperm to greater activity, while the other agglutinates the sperm, perhaps by so affecting the sperm-head surface as to cause it to

become more permeable to a viscous substance contained in the sperm, and given off into the sea water. Popa, '27, has recently described such a substance in *Arbacia* sperm.

TABLE XI.

PERMANENCE OF FOAM STRUCTURE IN SPERM SUSPENSIONS FOLLOWING RADIATION.

Length of Exposure.	*A.*			*B.*		
	Number of Drops of 1% Sperm Suspension per 100 cc. Sea-water.					
	30	15	5	30	15	5
0 min. . . .	××××	××	Scarce	××	Very few	Scarce
1 " . . .	××××	××	Scarce	××	Very few	Scarce
10 " . . .	××××	××××	××	×××	××	×
30 " . .	××××	××××	××	×××	××	×

A. Examined after 30 min.
B. Examined after 90 min.

Ultraviolet radiation likewise alters the permeability of the sperm surface, producing aggregates of sperm which, however, are not reversible, differing in this respect from the agglutinated masses produced by the action of normal egg-water upon normal sperm.

Certain of the above-described characteristics of fertilizin suggest an enzyme-like nature of the substance. Its high adsorption constant, the retention of some of its activity while in the adsorbed state, its displaceability by acids and not by fat-solvents, and an optimum pH range for activity and for adsorption have been described.

Tchachotine, '21, ascribed a sperm-agglutinating power to the gelatinous egg envelope. Although sperm do attach themselves in large numbers to the jelly layer of *Arbacia* eggs (if higher concentrations of sperm are used), this is probably due to the fact that the fertilizin is adsorbed by the jelly, and still retains some of its agglutinating power. A similar condition exists in the experiments described above, in which bits of charcoal with their adsorbed fertilizin behaved like artificial eggs, and attracted and held active sperm agglutinated in a film about them.

Glaser, '14*a*, found that sperm which had been paralyzed could

be reactivated, but not reagglutinated. Sperm treated with egg-water which had been strongly radiated become inactive, but may be reactivated by normal egg-water and to some extent reagglutinated. The sperm exudate had apparently not been entirely "fixed" by the egg-water, even though the sperm had become inactive. This is further evidence of the presence of two substances in egg-water, one responsible for activation, and the other for agglutination of sperm. Two other bits of evidence cited above also bear out this suggestion. Radiation may remove the agglutinating power of a given sample of egg-water, yet it may still retain its power to activate sperm. Also, egg-water adsorbed on charcoal does not lose its activating power, for when the charcoal and egg-water mixture is washed with sea-water, and the filtrate added to fresh sperm, the latter are activated but not agglutinated. Apparently the activating component of egg-water is not adsorbed by charcoal, or less so than the agglutinating component.

The colloid components of egg-water are best adsorbed within an optimum pH range which hovers about the point of neutrality. Recovery by acid is possible, and increasing the acidity of the egg-water beyond the optimum range, lowers its adsorbability by charcoal. This may depend on a reversal of the charge carried by the fertilizin particles, but further experimentation on this point is necessary. Saponin does not displace the fertilizin from adsorption. Radiation of fertilizin causes a slight increase in acidity. This is in accord with the findings of Stedman and Mendel, '26, for protein solutions and distilled water.

The echinochrome pigment in egg-water is faded by ultraviolet radiation. Its function is a rather uncertain one. Glaser, '21*a*, suggested that this substance acts as a stabilizer for fertilizin. It has been found to have a certain amount of photodynamic activity. (See R. S. Lillie and Hinrichs, '23.) The pigment is also adsorbed by charcoal, as shown in the above experiments, and by Glaser in 1921. (Glaser, '21*a*.) It is probably colloidal in nature.

The production of fertilizin by mature eggs has been discussed above at greater length. F. R. Lillie associates fertilizin production with viability of eggs as follows, "When an egg ceases to produce the sperm-agglutinating substance, it has lost its capacity

to be activated." (Lillie, '19, p. 240.) A reduction of fertilizin production following a temporary slight acceleration is probably due to the direct action of ultraviolet radiation upon the egg cortex. R. S. Lillie and Baskervill, '22, showed that radiation was effective in its direct action on the egg surface. Radiation induces membrane formation and fertilizin production is known to decrease as membranes begin to form.

Normal active sperm show an increased avidity for eggs which have been radiated a short time. Apparently this is due to the fact that ultraviolet radiation increases the permeability of the egg cortex, and liberates fertilizin to a greater degree than that found in normal eggs.

Normal sperm bear a substance necessary for fertilization which is given off into the medium, and after a certain period of loss of this substance, the fertilizing capacity of sperm decreases. Ultraviolet radiation augments the normal rate of loss of this substance from the sperm. (For a review of literature bearing on this subject, see Sampson, '26, and Hinrichs, '26*c*.) When radiation is long enough continued, sperm lose their capacity for normal fertilization as shown by the loss of ability of eggs, fertilized by them, to produce membranes, to cleave normally, and to develop into normal larvæ (Hinrichs, '26*b*). There is a corresponding loss of agglutinability of sperm by normal egg-water. Radiated sperm require a higher concentration of fertilizin to produce agglutination than do normal sperm. The same is true for sperm which have been standing for some time after removal from the gonad. (See Lillie and Just, '24.) Lillie and Just also report that the rapidity of onset of agglutination of sperm by means of normal sea-water is a function of the motility of the sperm. Radiation reduces sperm activity and also lengthens the time of onset of agglutination.

Radiation produces an increase in the viscosity of sperm suspensions. Mond, '22, and Wels, '23, report similar findings for the effects of ultraviolet and x-radiation upon proteins. In radiated sperm, this increase in viscosity, together with a reduction in motility which follows, is probably responsible for the permanence of clumps which are formed as the result of radiation alone. When normally agglutinated masses are radiated, the clumps be-

come permanent, due to the increased adhesiveness and loss of motility of individual sperm. Also, radiated sperm produce reticular, stringy masses on the addition of normal egg-water. Here again, the increased stickiness of the sperm and the loss of motility are a result of radiation.

I wish to acknowledge my gratitude to Dr. R. S. Lillie for his coöperation in the study of this problem. The experiments were made at Woods Hole during the summer of 1926.

Conclusions.—I. Earlier work with ultraviolet radiation and *Arbacia* germ cells has shown the following results: (*a*) Radiated eggs fertilized by normal sperm, normal eggs fertilized by radiated sperm, or radiated zygotes produce differentially modified larvæ, (*b*) Radiation of normal sperm causes a reduction and loss of motility and fertilizing power, and (*c*) Radiation of normal sperm suspensions causes sperm to form irregular, permanent aggregates. Continued radiation kills the sperm.

II. The present experiments have added the following:

(*a*) The radiation of normal eggs produces at first a slight increase, then a decrease, and finally a complete loss of the power of producing fertilizin. There is a parallel loss of viability of the eggs as measured by their fertilizability and ability to develop normally.

(*b*) The radiation of normal egg-water produces a fading of echinochrome pigment, and a reduction of the agglutinating power of fertilizin. The two are affected at different rates. The additive effect of temperature and radiation is more rapid in its action upon fertilizin, than upon echinochrome.

(*c*) The agglutinating power of egg-water is lost before its sperm-stimulating power, suggesting that two distinct substances may be concerned.

(*d*) There is an optimum pH range, around the point of neutrality, for the agglutinating action of egg-water. A similar range is present for the adsorption of fertilizin by charcoal. Adsorbed fertilizin retains some of its agglutinating power. The enzyme-like character of fertilizin is indicated. Adsorbed fertilizin may be displaced from adsorption by a slight acidification, but not by the addition of surface-active compounds. Fertilizin has a high adsorption constant. For this reason, fertilizin is prob-

ably adsorbed by the egg surface to an extremely high degree, and usually not enough of it is present in an active state in the spaces between eggs to produce typical agglutinated masses of sperm, when eggs are inseminated in fingerbowls in the laboratory.

(*e*) Ultraviolet radiation produces a slight increase in the pH of egg-water.

(*f*) Sperm suspensions become more translucent as a result of radiation, in consequence of the formation of permanent irregular aggregations of sperm which settle out of suspension.

(*g*) The more concentrated suspensions of sperm show an increase in viscosity and a decrease in surface tension following radiation. The increase in viscosity is associated with the formation of aggregates of sperm.

(*h*) Radiated sperm undergo a reduction of agglutinability by normal egg-water. The onset of agglutination is delayed, and the duration of the phenomenon is shortened.

(*i*) The more dilute suspensions are more susceptible to radiation effects.

(*j*) Radiation effects with eggs, egg-water, and sperm are graded by dosage of radiation.

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GENETIC EVIDENCE FOR DIPLOID MALES IN *HABROBRACON*.[1]

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The production of males from fertilized eggs in the parasitic wasp, *Habrobracon juglandis* (Ashmead), is discussed in a previous paper (Whiting, Anna R., 1925). It was shown that homozygous orange-eyed females, when crossed to related males with dominant black eyes, produced a few practically sterile black-eyed sons, in addition to the usual number of impaternate orange-eyed males and heterozygous black-eyed daughters. The few daughters of these " patroclinous " or biparental males were black-eyed, almost completely sterile and in many cases abnormal in appearance. It was postulated that a spermatozoön developing independently in the egg cytoplasm and crowding out the egg nucleus might produce a male which, although resembling its diploid sisters, would be haploid. This did not explain why recessive males failed to appear in broods from the reciprocal cross of homozygous black females by orange males; neither did it throw any light on the sterility of the biparental males and of their few daughters.

With the occurrence of new mutations additional facts have been brought out consistent with previous results and throwing further light upon the problem.

A series of quadruple allelomorphs affecting eye color (Whiting, Anna R. and Burton, Raymond H., 1926) has been used in various combinations in breeding experiments to be discussed. These are black (O), light (o^l), orange (o), and ivory (o^i), in decreasing order of dominance. In black, orange, and ivory the ocelli are of the same color as the compound eyes. In light, they are gray while the compound eyes remain black. Distinction from

[1] The author is indebted to Bussey Institution, Harvard University, for the use of space and equipment, and to the Committee for Research in Problems of Sex of the National Research Council for financial aid in carrying on this work.

type is more easily made in females than in males and is especially clear in light females heterozygous for orange or ivory.

Three pairs of allelomorphs affecting the wings have also been used. Wrinkled (w), recessive to type (W), prevents complete expansion of the wings and often the normal development of legs and antennæ. It is almost completely recessive and somewhat lethal in the homozygous and azygous conditions. Reduced (r), completely recessive to type, reduces the wings, especially the primaries, in size and venation. No overlapping with type occurs. These mutations are discussed at length elsewhere (Whiting, P. W., 1926). Defective (d) (referred to in previous publication as d_{1195}, Whiting, P. W., 1924), like wrinkled, is almost completely recessive but shows some overlapping with type (D). It reduces the length or causes disappearance of the fourth branch of the radius vein (r_4) in 90–95 per cent. of pure stock under standard conditions. Unfortunately reduced and defective cannot always be recognized with certainty in wrinkled, while defective cannot be identified in reduced where the veins are so generally disarranged.

There is no linkage between any of these factors.

Crosses Producing Biparental Males.

Table I. gives summaries of crosses which produce biparental males. Whenever significant differences involving locus D occur they are indicated in the formulæ. The impaternate haploid males arise from unfertilized eggs and resemble their mothers. Where the mothers are heterozygous these males fall into two classes as expected. Females show all the dominant traits of both parents as do the biparental males.

The first cross in the table will serve to illustrate this. Mothers are light, fathers black. All daughters are black, most sons light, the few biparental sons black.

In classes c and e, section I., mothers are defective (d), fathers normal (D). If numbers in these classes be combined it is found that 6 of the 58 biparental males are defective, 10.34 ± 2.70 per cent.; 753 of the 820 impaternate males, 91.83 ± 0.64 per cent.; 46 of the 569 females, 8.08 ± 0.77 per cent. Similarity of percentages in biparental males and females is apparent. The 13

light males produced in classes d and e give still further evidence of biparental inheritance. Their mothers were orange or ivory, fathers light. They resemble their heterozygous sisters as their ocelli are lighter in color than those of males from light stock.

Table I.

Crosses Producing Biparental Males.

Section.	Parents.		Matings	Progeny.		
	♀.	♂.		Biparental ♂♂.	Impaternate ♂♂.	♀♀.
I *a*	o^1o^1	O	10	7	156	115
b	oo	O	246	489	11,221	8,436
c	o^1o^1dd	OD	32	55	764	544
d	oo	o^1	8	10	276	326
e	o^io^idd	o^1D	1	3	56	25
f	o^io^i	o	11	32	744	545
II *a*	OOrr	OR	4	8	93	137
b	oorr	oR	4	5	47	54
c	o^io^irr	o^iR	9	9	45	92
III *a*	o^io^irr	o^1R	1	3	4	22
b	o^io^irr	oR	9	5	39	94
c	o^1o^1RR	Or	17	17	392	393
d	ooRR	Or	26	30	758	808
e	o^io^iRR	Or	2	3	119	110
f	OOrr	oR	3	1	16	35
g	OOrr	o^1R	1	5	11	15
h	oorr	o^iR	6	8	82	78
IV *a*	ooddww	ODW	3	5	43	93
b	ooWW	Ow	9	19	643	449
c	ooddWW	ODw	14	29	660	404
d	o^io^iddWW	ODw	19	17	637	360
e	ooWw	OW	1	13	79	77
V *a*	ooWWrr	OwR	5	7	63	83
b	ooDDWWrr	OdwR	3	3	6	26
c	ooddWWrr	o^iDwR	3	4	17	70
d	oo^iWWrr	owR	3	3	41	48
Total			450	790	17,012	13,430

In section II. are data on crosses where parents differ in wing character, locus R, but are similar in eye color. Biparental males can be readily recognized by their non-reduced wings. These are of interest since they show that the chromosome carrying R acts in the same manner as those containing O and D.

In section III. are given summaries of crosses involving differences in O and R. Whether one or two recessive factors are present in the mother the biparental males show both dominant characters.

Section IV., deals with crosses involving differences in the O and W loci, and in some cases in D. Cross a, ooddww females by ODW males, gave 5 black-eyed males 1 of which was wrinkled. All had normal venation. Since homozygous wrinkled females have bred true in all cases tested by the author there is little doubt that the W is contributed by the father. All orange sons were wrinkled, practically all that could be identified were defective, and all black daughters normal as to wings.

Wrinkled sometimes occurs as a result of accident of growth. Since biparental males are abnormal in so many ways, as for example in their rather common habit of pupating without spinning a cocoon, their wings might have a greater chance to be wrinkled, thereby increasing percentage of wrinkled in this class.

Cross c, section IV., ooddWW female by ODw male, is of importance. A dominant factor affecting the wings is contributed by each parent. Of the 29 black non-defective sons, 25 were non-wrinkled while 4 had slightly wrinkled wings, a condition parallel with that found in the sisters except that percentage of wrinkled is higher. There was one wrinkled among the 660 orange males and 3 wrinkled among the 404 black females. Class d resembles this except that ivory females were used instead of orange. Results are similar. Nineteen matings gave 17 black males, 1 with wrinkled wings and 1 with defective venation, 637 ivory males, practically all defective, and 360 black females, 1 with wrinkled wings.

In section V. are types of crosses involving differences in O, W, R, and in two cases D. In class a, ooWWrr females by OwR males, there were produced 7 black, non-wrinkled, non-reduced males and 63 orange non-wrinkled reduced males. The females were like the biparental males except that 1 defective appeared. In class b the four differences are involved, two dominant factors contributed by each parent. The 3 biparental males were entirely dominant, the orange sons non-wrinkled and reduced, the daughters dominant except for two defective individuals. Wings of

biparental males show three dominants, two from the mother, one from the father.

Class c is likewise of special interest. Recessives d and r are contributed by the mother, o^{i} and w by the father. Biparental males are orange, non-defective (with one exception slightly so), non-wrinkled, and non-reduced. Here again the wings of biparental males show three dominant characters, two from the father and one from the mother. In class d three females heterozygous for o and o^{i}, homozygous for W and r, are mated to owR males. All ivory males have reduced wings while among the orange males are the biparental males easily recognized by their non-reduced wings.

In all, four hundred and eighteen matings were made where female parent had recessive eye color, male dominant. 747 biparental males were produced in addition to 16,660 impaternate males and 12,901 females.

One hundred and thirty-five matings of defective females by non-defective males gave 307 biparental males, 6,325 impaternate males and 3,816 females. Defectives were recorded in one hundred and two of these matings. 30 defectives were found among 269 biparental males, 11.15 ± 1.29 per cent.; 4,744 among 5,159 impaternate males, 91.95 ± 0.25 per cent.; 175 among 3,085 females, 5.67 ± 2.81 per cent.

Three matings of ww females by W males gave 5 biparental males, 1 wrinkled, 43 impaternate males, all wrinkled, and 93 normal females.

Fifty-one matings of rr females by R males gave 61 biparental males, 462 impaternate males and 754 normal females.

Tests of Biparental Males.

Table II. gives summary of tests of biparental males. Whenever possible they were tested by mating to homozygous recessive females. Individual males were often mated to several females. Since they produce but few daughters the results of a large amount of work seem meager.

Two hundred and forty-two males were tested. One hundred and ninety-seven or 81.40 ± 1.69 per cent. were found to be sterile. Of these, one hundred and thirty-nine were tested once,

forty-five twice, six three times, four four times, two five times, and one seven times, in all two hundred and eighty matings resulting in 23,089 sons, no daughters.

TABLE II.

TESTS OF BIPARENTAL MALES.

Probable Formulæ of ♂♂.	Source (See Table I).	Sterile ♂♂.	Sons of Mates.	Fertile ♂♂.	Daughters.	Sons of Mates.
Oo^1.....	I *a*	4	396	0		
Oo.....	*b*	145	18,692	31	104	3,769
Oo^iDd.....	*c*	4	145	0		
o^1o.....	*d*	2	56	1	5	38
o^1o^iDd.....	*e*	1	72	0		
oo^i.....	*f*	7	654	3	13	750
OORr.....	II *a*	1	159	1	21	66
ooRr.....	*b*	3	265	0		
o^io^iRr.....	*c*	7	346	1	1	25
oo^iRr.....	III *b*	1	55	1	6	9
Oo^iRr.....	*c*	5	137	2	6	160
Oo^iRr.....	*g*	1	56	2	25	816
oo^iRr.....	*h*	4	323	3	5	296
OoDdWw...	IV *a*	5	1,010	0		
OoDdWw...	*c*	4	705	0		
OoWW(w)...	*e*	3	18	0		
Total.....		197	23,089	45	186	5,929

Of the forty-five fertile males twenty-six were tested once each, seven twice each, four three times, two four times, three five times, one seven times, and two ten times, one hundred and two matings. Altogether these matings gave only 186 daughters among the 5,929 sons of the females, an average of 3.135 daughters for each fertile biparental male. Seven matings of one male made at two day intervals resulted in 21 daughters, 15 of these in one mating. Another male mated ten times at two-day intervals gave in five matings 21 daughters. These are the most prolific by a rather wide margin. Results indicate that more daughters could be obtained from biparental males by making repeated tests.

With two exceptions all biparental males produced daughters showing the dominant characters like themselves. They therefore breed like haplonts. This was true of the D, O, and R loci irrespective of the side from which the factors came. Although

nine biparental males carrying w were tested by crossing to ww females, some of them several times, no daughters were obtained among the 1,733 sons, all wrinkled like their mothers.

Daughters of Biparental Males.

Of the two exceptional biparental males mentioned above one occurred in a mating of ivory defective female by orange defective male. He had orange eyes and defective wings as would be expected. His mate was of the same genetic constitution as his mother. In the first culture bottle appeared 2 orange females, 1 with abnormal abdomen which died within the cocoon, the other sterile. In the fourth bottle was found an ivory defective female. She was fairly fertile. In the first culture bottle she laid over thirty eggs, one of which hatched into an ivory female. In the second bottle there was likewise a high mortality of eggs but 9 ivory males and 5 ivory females ultimately emerged. Her daughter from bottle a gave 49 males, another which lived but a short time gave 10 males. There are three possible explanations for the appearance of this ivory female. Her mother may not have been virgin when mated. This is improbable since great care was exercised in this matter and many more ivory females would be expected early in the life of the mother if she had mated with an ivory brother. The female may have been produced from an unfertilized egg, a phenomenon which has occurred but rarely in *Habrobracon,* or the male may have produced a spermatozoön not carrying the chromosome containing the o factor.

The comparatively late appearance of this female serves as an argument for the second explanation since daughters of biparental males usually appear in the first or second culture bottles. On the other hand the high mortality of her eggs might indicate that she is the daughter of the biparental male since the few females obtained from unfertilized eggs have proved highly fertile.

The second exceptional case was found early in the work by P. W. Whiting. It has not been previously discussed in detail. A male of type stock 1 was crossed with female of orange defective stock 3. 118 orange sons showed defect typical of stock 3. Black daughters were 15 normal, 1 defective; black sons were 3 normal, 1 defective, the defective among the black being due to

irregular dominance. One of these normal black males was mated to three orange females by each of which he produced a single daughter.

From one cross there were produced 30 orange males and in vial b a single black-eyed female of small size and with asymmetry of ventral abdominal sclerites. She produced only one larva which died. Whether she posessed D or d is not known. Her morphological abnormality and near sterility are comparable with such conditions in daughters of biparental males transmitting only dominant traits.

From another cross there were produced 92 orange males and in vial c a single orange female. She developed from a naked pupa and had asymmetrical sternites. She appeared normal in internal morphology and histology and produced 3 orange sons, 1 normal and 2 defective. Since her mother was of normal orange stock the occurrence of these indicates that she had received d from her father. Like many daughters of other biparental males she was of abnormal appearance, but unlike them she was somewhat fertile.

From the third cross there were produced 136 orange males and, in vial a, a single orange female. This female was normal in appearance and produced 71 offspring, males 13 normal, 16 defective, and females 23 normal and 19 defective. The defectives were due to the fact that this female's mother had d. Normal venation was isolated in later generations.

This biparental male had, therefore, in addition to black-bearing spermatozoa, two types of orange, od, the maternal combination, and oD, a recombination type. He is the only male found which breeds like a heterozygote. He and his daughters are not included in the following summary.

Of the 186 dominant daughters of biparental males only 121 were sufficiently normal to test. Most of these laid eggs which failed to hatch. Some lived for several days, stung the host caterpillars but laid no eggs. One gave a normal black male which proved sterile; one produced a black-eyed female pupa which died in the cocoon; one gave a morbid larva which died young and an abnormal female pupa with black eyes found dead in the cocoon; and another an abnormal pupa of uncertain eye-color and sex, and a fifth *five* larvæ which died and a female pupa of uncertain eye-

color. The dominant daughters have so far given only dominant offspring.

Morphological Abnormalities in Biparental Males and their Daughters.

Physical defects are rather common in biparental males and their daughters. These include abnormal sclerites in abdomen, defects in antennæ, abnormal legs, incomplete digestive tract, abnormal thorax, and genitalia. Among the 790 biparental males were found 41 or 5.19 ± 0.53 per cent. abnormal. Also there were 61 individuals that did not spin cocoons (called naked pupæ) 7.72 ± 0.64 per cent. This stands in contrast to conditions in impaternate males where there were but 24 abnormal and 83 naked pupæ among 17,111 normal, 0.14 ± 0.02 per cent. and 0.48 ± 0.04 per cent. respectively. Among the 13,430 sisters of biparental males there were 82 abnormal and 57 naked pupæ, 0.61 ± 0.04 and 0.42 ± 0.04 per cent. respectively.

In the daughters of biparental males abnormalities are often more extreme and present in a much higher percentage. 68 freaks and 23 naked pupæ appeared in addition to 95 normal. Percentage of freaks is 36.56 ± 2.38, of naked pupæ 12.36 ± 1.63. Brothers of these females showed 13 freaks and 2 naked pupæ to 5,914 normal, 0.22 ± 0.04 and 0.03 ± 0.02 per cents.

Summary.

1. Four allelomorphs affecting eye color and three pairs of allelomorphs affecting wing form and venation, none linked, are studied from the point of view of the method of their inheritance by biparental males in *Habrobracon juglandis* (Ashmead).

2. A female homozygous for one or more recessive factors when crossed to a male carrying allelomorphs to these factors produces, in addition to recessive haploid sons and dominant diploid daughters, sons which have all dominant characters like their sisters.

3. In crosses where females are homozygous for some recessive and some dominant factors and males possess allelomorphs the biparental sons are entirely dominant, showing that they have some factors from each parent.

4. When three of these factors affect one structure (the wing in this case) if one recessive and two dominants are contributed by one parent, their allelomorphs by the other, this structure in biparental males shows all the dominant characters.

5. From these results it is concluded that biparental males are diploid (Whiting, P. W. and Whiting, Anna R., 1925) at least for the four chromosomes that can be identified genetically.

6. Biparental males and their daughters are often abnormal in appearance and usually sterile or nearly so. When fertile they breed as dominants (with one, and possibly two, exceptions noted above).

Discussion.

When different types of crosses are made between inbred related stocks and results summarized it has been found that definite relationships exist between various percentages derived from these summaries.

Percentage of males among biparental offspring, (previously called percentage of patrocliny), $\frac{\text{biparental } ♂♂ \times 100}{\text{biparental } ♂♂ + ♀♀}$, is negatively correlated with percentage of females, $\frac{♀♀ \times 100}{\text{total,}}$, and with percentage of total biparentals, $\frac{\text{biparental } ♂♂ + ♀♀ \times 100}{\text{total}}$. In other words when a type of cross results in a high percentage of males among biparentals there is a low percentage of females and of total offspring from fertilized eggs. The female percentage is lower than it would be if the decrease were due only to the transformation of some fertilized eggs into biparental males. This indicates that there is a mortality of fertilized eggs in these crosses directly correlated with number of biparentals which are males. Types of crosses that produce no biparental males have the highest percentage of females.

The question as to why these individuals are males cannot be answered at this point. Dr. Castle has suggested that they may correspond to the intersexes which Goldschmidt gets in *Lymantria* (Goldschmidt, Richard, 1927). Some of the facts support this and it may be that the presence of certain genetic factors in some individuals causes sex reversal. No gradations have been ob-

served in *Habrobracon*. The biparental males have all been completely male in external and internal morphology and in reactions. The high mortality suggested above may be due to intergrades that cannot survive in this species. In *Lymantria* the intergrades occur as a result of wide crosses while in *Habrobracon* these males come from crosses of related stocks only.

Non-disjunction may also be considered a possibility. Biparental males may be diploid for all chromosomes save one, the sex chromosome, and thus be males although resembling their sisters in appearance.

Unfortunately *Habrobracon*, like the Hymenoptera in general, is not ideal for cytological study. Haploid number of chromosomes seems to be eleven and they are extremely minute. Nachtsheim has demonstrated (Nachtsheim, H., 1913) that in the honey bee chromosomes frequently fractionate so that somatic counts vary considerably being various multiples of the haploid number. The author finds indications of this in another form now under observation so that a very careful study and numerous counts are necessary before conclusions can be drawn.

In spermatogenesis of biparental males the first maturation division is abortive, the second apparently equational as in normal haploid males. This process may result in diploid spermatozoa which when united to recessive eggs would give only dominant triploid offspring. The high percentage of physical abnormalities in daughters of biparental males and their sterility may be due to their triploidy. They possess ovaries and ova normal in general appearance.

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CARBON DIOXIDE AS A NARCOTIC AGENT.

I. The Effect of Carbon Dioxide upon the Fertilized Egg of *Arbacia*.

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The narcosis which may be produced by carbon dioxide seems to have long been known. Pliny the Elder, in his Natural History, remarked that the marble from Memphis, when ground up and used as a liniment with vinegar, had the virtue of rendering insensible parts of the body to be cut or cauterized. In modern times the value of carbon dioxide as an analgesic was recognized soon after Black's discovery of "fixed air," and in 1788 we find Percival recommending "fixed air" for the relief of painful ulcers. Later it was employed as a local anesthetic by Ingenhousz,[1] Beddoes, Simpson, Follin, Brown-Séquard,[2] Gellé,[2] and others. In 1828, eighteen years before Morton's demonstration of ether anesthesia, Hickman[3] appears to have suggested that general anesthesia be induced by inhalations of carbon dioxide, and it was thus used later in the century by Ozanam and by Gréhant, although never extensively. Ozanam seems to have been impressed by the promptness of recovery from the anesthesia produced in this way. At one time it was thought by some that natural sleep and hibernation were the effects of the accumulation of carbon dioxide. This view has been abandoned, but Kidd (1914) has recently with more plausibility suggested a similar explanation for the dormancy of seeds. The work of Cohn (1918) also indicates the possibility that carbon dioxide may play an important part in keeping the spermatozoa of *Arbacia* inactive until their discharge into sea water.

[1] Cited by Herpin (1864).
[2] Cited by Dastre (1890).
[3] Cited by Simpson (1856).

Inasmuch as carbon dioxide is a normal product of metabolism, an understanding of its characteristic effects on living cells and the mechanism by which they are produced is of importance. Unfortunately, most of the studies previously made on its action have been concerned with entire organisms where numerous complicating factors prevent the separation of its more characteristic effects from others of a less fundamental nature. It is the object of the present paper to study in a quantitative way certain aspects of the narcotic effects of carbon dioxide on single cells—the developing egg cells of *Arbacia*—where complicating factors are reduced to a minimum. In particular, an attempt has been made to determine the extent to which such effects are reversible. A second paper [1] deals similarly with a simple tissue—the striated muscle of the frog—and studies upon ciliated epithelium are to be reported elsewhere.

For quantitative work, the developing egg of *Arbacia* proves very satisfactory. Since favorable material shows about 100 per cent. division under normal conditions, any delay in the occurrence of the first cleavage may be measured and used as a suitable quantitative criterion for narcotic or toxic effects upon the activity of the cell. Smith and Clowes (1924) have studied from a somewhat different point of view from that of the present paper the effects of carbon dioxide upon the cleavage of echinoderm eggs and have found that development is inhibited by CO_2 at pH values which in its absence are without effect upon cleavage. The present experiments differ in at least two respects from those of Smith and Clowes. In the first place, they have to do with exposures of varying lengths *followed by a return to normal sea water* to permit a determination of the extent to which the effects produced are reversible. In the second place, a different method has been used for the quantitative measurement of the degree of retardation of the developmental processes. Instead of measuring the total number of cell divisions secured in a given time irrespective of whether they be the first, second, or third, the quantity here measured has been the time required for the first cleavage in each case. Furthermore, this has been done in such a way as to take into account

[1] *Amer. Journ. Physiol.*, LXXXII., 241.

not merely the mean time for all of the eggs but, approximately at least, the time for each individual egg. It is believed that data of this sort, while more troublesome to secure, are theoretically more significant and have a wider range of usefulness than those obtained by the other method.

Method.

Sea water, saturated with CO_2 from a Kipp generator, was used directly or was diluted as desired with oxygen-saturated sea water, with nitrogen-saturated sea water, or with ordinary sea water by siphoning together appropriate amounts of these solutions. It was then immediately siphoned into 75 cc. glass-stoppered bottles containing a few drops of a concentrated suspension of the newly fertilized eggs of *Arbacia punctulata*. As soon as the bottles were completely filled, they were tightly stoppered, shaken briefly, and placed in a bath of running sea water, which, with a few exceptions, varied in temperature not more than 0.5° C. for the duration of each experiment. For all the experiments during one season the range of temperature was 18.4° C. to 22.4° C., with a mean value of 20.3° C. In order to prevent a change in the solubility of the dissolved gases, it was deemed important always to have the CO_2-containing solutions at the temperature of the water-bath. Care was also taken to begin all the exposures of any one series as nearly simultaneously as possible, since a number of experiments not reported here seemed to indicate that sensitiveness to CO_2 may vary prior to the appearance of the first cleavage. In many of the experiments the bottles were inverted at five minute intervals to keep the contents well mixed, but other experiments in which this precaution was less rigorously observed gave essentially the same results.

At the time of setting up an experiment, samples of the solutions used were taken for estimations of the pH and dissolved oxygen. The former were immediately determined with the use of phenol red, brom thymol blue, brom cresol purple, and methyl red as indicators; the samples for the latter were kept at a constant temperature until Winkler determinations could be made. The results of the Winkler determinations are expressed as cc. of

oxygen per liter. Otherwise, the oxygen content is stated, as is the carbon dioxide content, in terms of percentage saturation. Assuming the applicability of Henry's law to gases in solution, it may be said that when 20 cc. of oxygen-saturated sea water is added to 80 cc. of CO_2-saturated sea water, the resulting solution contains CO_2 at 80 per cent. of saturation value and oxygen at 20 per cent. of saturation value. Since in adding the saturated solutions to the eggs they must experience a small interchange of gases with the air, a solution which was initially free of oxygen is referred to as having a trace of oxygen, while a CO_2-saturated solution is represented as having a CO_2 content of "100 —" per cent. Wherever the tension of CO_2 is expressed in mm. Hg the value given is merely an approximate one, calculated for purposes of comparison with the work of other investigators on the assumption that the tension of carbon dioxide in a saturated solution is 760 mm., minus the vapor pressure of water at the temperature in question.

As already mentioned, the reversibility of the effects of CO_2 was determined by returning the eggs to sea water for development after the desired periods of exposure. Following a rinsing in sea water, the eggs were placed in small Pyrex beakers containing sea water to a depth of about 1.5 cm. Samples of the eggs were removed from the beakers at various times and were preserved for subsequent observation by the addition of a weak solution of formalin in sea water. The fixed eggs were placed in a large hanging drop where, if free from debris, they tended to settle in rows, which simplified the task of determining the percentages of eggs which had undergone the first cleavage. These values, found from time to time after a given exposure, give, when plotted against the minutes after fertilization, a curve which will be referred to as the cleavage curve for that particular exposure. The characteristic S-shape of this curve is related to the variability of the eggs themselves in the manner discussed by Loeb and Northrop (1917) and by Brooks (1918). The time required for cleavage in 50 per cent. of the eggs has in these experiments been used as the most convenient criterion of the cleavage rate, but other percentages could equally well be compared; the time in question can,

of course, be readily found by interpolation from the cleavage curve.

Results.

Before discussing the typical effects of carbon dioxide, it is necessary to rule out the possibility that oxygen lack might be a contributing factor in the results produced, since the method used in saturating the sea water with carbon dioxide causes at the same time a removal of oxygen. Although a complete lack of oxygen has been shown to stop the cleavage process in sea urchin eggs (E. B. Harvey, 1927), the present work indicates that even with an extensive reduction in oxygen tension cleavage is able to continue—and at a rate but little slower than normal. The results of the thirty minute exposures to low tensions of this gas, representing but 14 per cent. to 18 per cent. of those available for the controls, are given in Table I. and show that the cleavage time under these conditions was delayed but a few minutes.

Table I.

The Effect upon Cleavage of Thirty Minute Exposures to Low Oxygen Tensions.

Solution.	cc. of Oxygen per Liter.	Minutes Required for 50 per cent. Cleavage.
Sea water	5.6	60.5
Nitrogen-saturated sea water	0.76	68.5
Sea water	5.48	62.5
Nitrogen-saturated sea water	0.97	64.2

A direct comparison of the effects of low oxygen tension and of high carbon dioxide tension has been made in another experiment, in which each of four portions of egg suspension was exposed for thirty minutes to one of the following solutions:

	pH.	cc. of Oxygen per Liter.
1. Sea water	—	5.15
2. Sea water + oxygen + CO_2 (60% saturated)	5.3	5.56
3. Sea water + nitrogen + CO_2 (60% saturated)	5.3	1.26
4. Sea water, saturated with nitrogen	—	0.55

It will be evident that solutions 3 and 4 were low in oxygen as compared with solutions 1 and 2, while solutions 2 and 3 were high

in CO_2 as compared with solutions 1 and 4. The first cleavage appeared as shown in Fig. 1, where it will be seen that the shortest cleavage time occurred with the sea water control, represented by Curve 1. A lowering of the oxygen tension to slightly over one tenth of the normal value retarded the cleavage time but 4 minutes

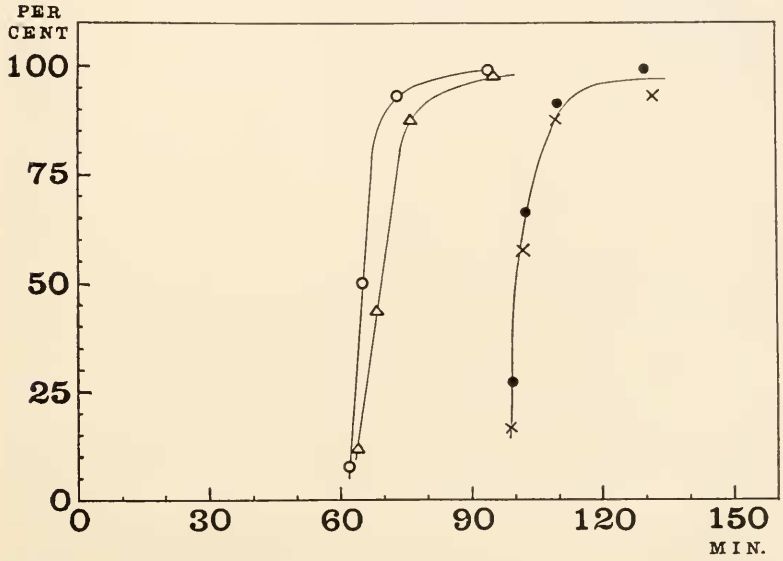

FIG. 1. The relative effectiveness of low oxygen tensions and high CO_2 tensions in delaying the first cleavage of *Arbacia* eggs. Exposures were of thirty minutes' duration. Temperature, 19.8°–20.2°. Abscissa = time, in minutes, after insemination. Ordinate = percentage of eggs showing the first cleavage.

	Symbol.	cc. Oxygen. per Liter.	CO_2.
Curve 1	○ Circles	5.15	
Curve 2	× Crosses	5.56	60%
Curce 3	● Dots	1.26	60%
Curve 4	△ Triangles	0.55	

(Curve 4). On the other hand, an increase of the CO_2 content to give pH of 5.3 was sufficiently great to retard the cleavage time 36 minutes, irrespective of the oxygen present, since a single curve serves to represent cleavage in the two solutions which, although showing more than a 4:1 difference in oxygen content, had the same carbon dioxide content. Since from such experiments as

the foregoing the cleavage process is seen to be but little affected by oxygen deficiency over a wide range, the more striking effects observed with CO_2, about to be described in more detail, may justly be attributed to an action of carbon dioxide in which incidental oxygen lack plays little or no part.

Such being the case, the solutions used in the subsequent experiments were simply mixed in a large graduated cylinder and immediately added to the eggs. Siphons were not used nor were Winkler determinations made. Speed in setting up the experiment was important, and in cases where a considerable number of bottles were to be filled, this method had the advantage of expediency. Where the sea water used was saturated with CO_2 (i.e. "100 —" per cent. CO_2, with a trace of oxygen) it seems probable that sufficient oxygen must have entered the solution from the air to prevent any retardation of cleavage from oxygen lack, since a 4:1 mixture of CO_2-saturated and oxygen-saturated sea water (*i.e.* 80 per cent. CO_2 and 20 per cent. oxygen) gave practically the same result. In fact, the results following "100 —" per cent. CO_2 were very similar to those of various tensions down to as low as about 30 or 40 per cent. CO_2. Inasmuch as most of the work here reported is not concerned with more than a semi-quantitative estimate of the gases, it is believed that the method is sufficiently accurate.

In striking contrast to the extensive diminution in available oxygen which the eggs seem to tolerate is the effect upon cleavage of even very small amounts of carbon dioxide. The repression of cleavage which occurs when, in the laboratory, the eggs are subjected to overcrowding is a familiar example of the effects which may be produced simply by the CO_2 which arises from the metabolism of the cells themselves. Experimentally, sea water containing as little CO_2 as 10 per cent. of saturation value was found, after an exposure of twenty minutes to delay cleavage twelve and one half minutes, while exposures of equal length to 30 per cent. and 40 per cent. CO_2 were found to delay cleavage a longer time—about twenty-three minutes. Apparently this latter value of 40 per cent. saturation or 300 mm. Hg represents practically a complete suppression of cleavage since it is approximately the same value as is obtained with higher tensions.

TABLE II.

PERCENTAGE OF EGGS CLEAVED DURING EXPOSURE TO CO_2.

Experiment.	No. 130.	No. 131.	No. 129.	No. 120.
Temperature	22.3°	21.7°–22.1°	21.3°–22.4°	21.2°–21.6°
Per cent. CO_2	15%	20%	30%	80%
pH	*6.25*	*6.2*	*5.05*	*5.2*
Exposures (in minutes):				
80′	10.9%	0%		
90′	27.7%			
100′		1%	0%	
120′			0%	
140′	56.8%			
160′			0%	
200′			6.8%	
300′			43.8%	
640′				0%

It is evident from Table II. that with less than 30 per cent. saturation the repression of cleavage is not complete, but only partial. At a value of 30 per cent., repression is almost complete, since only 7 per cent. and 44 per cent. of the eggs were able to cleave in three and one third and five hours respectively. It will be shown later in the paper that the effects of 30 per cent. and 40 per cent. CO_2 are so similar to those produced by greater amounts, that it is reasonable to suppose that at a saturation of 40 per cent. and over, representing a tension of 300 mm. Hg or more, the suppression of cleavage is practically complete. Therefore not only can very small amounts of CO_2 in the surrounding medium cause an appreciable delay in cleavage, but also its maximum action in suppressing cleavage is approached at values far below saturation.

Notwithstanding the prompt and extensive check upon cleavage which carbon dioxide produces, the reversibility of its effects upon returning the eggs to sea water serves to indicate that the action has been of a narcotic rather than of a purely toxic nature. After exposures of twenty minutes to sea water practically saturated with CO_2 there are usually no abnormalities in cleavage, and larvæ develop which show little or no difference from the normal controls. Following longer exposures, abnormalities appear, although they are relatively few in number with exposures of less than an hour. In spite of the presence of many abnormally

cleaved eggs, a few ciliated larvæ have been found to develop after an exposure of two and one half hours, and the first cleavage has made its appearance in 95 per cent. to 100 per cent. of the eggs subjected to very nearly 100 per cent. CO_2 for this length of time, though relatively few normal larvæ were obtainable after such long exposures. In one experiment—that illustrated in Table III.—exposure of the eggs to 80 per cent. CO_2 for over ten hours still permitted reversibility of the cleavage process to the extent that, six hours after the eggs had been returned to sea water, 88 per cent. were found to have divided, although development was very abnormal and went no farther than the first few cleavage stages. Apparently the process of nuclear division is an extremely powerful one and, as has been observed by others, can persist even when the cell itself is unable to divide.

Fig. 2 is a typical illustration of the series of cleavage curves which are obtained with various lengths of exposure to a relatively high tension of carbon dioxide. It will be observed that the first cleavage ultimately occurs in practically all of the eggs, even after prolonged exposures. The relation of the exposure time to the total retardation is a matter of some importance in indicating the nature of the observed effects. If the narcosis produced by carbon dioxide were complete and were followed by instantaneous recovery the resulting retardation of cleavage, as compared with normal controls, should be exactly equal to the time of exposure. Incomplete narcosis, on the other hand, would tend to shorten, and a more gradual recovery, to lengthen, the period of retardation. Theoretically, a combination of these two effects might conceivably, under a given set of conditions, result in a net retardation exactly equal to the period of the exposure, but it is believed that such a balancing of effects could not account for the results about to be described. Since with all tensions above approximately 30 per cent. saturation the relation of time of exposure to total retardation is essentially the same, it is difficult to imagine that the CO_2 tension should, above this point, either be without effect on a measurable rate of development or that a decrease in this rate should always be followed by a correspondingly increased rate of recovery. The reasonable interpretation of the

facts is that development is practically suppressed by the higher tensions of CO_2. The correctness of this view is also indicated by the fact that cleavage was not obtained during prolonged exposures to such solutions.

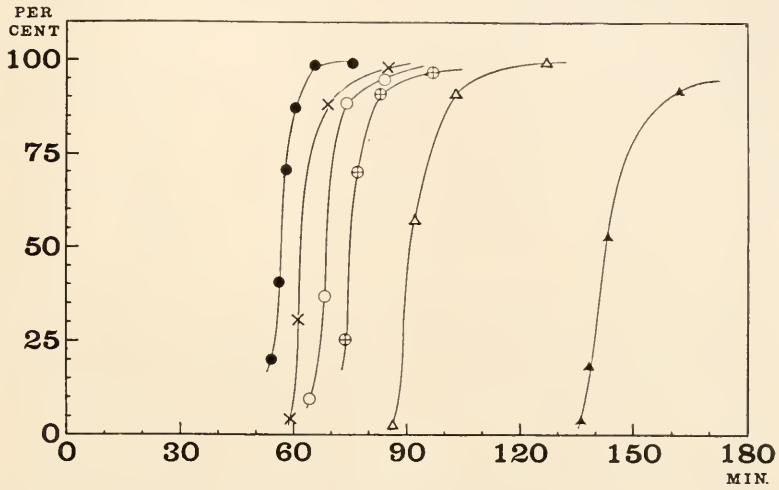

FIG. 2. The times required for the appearance of the first cleavage of *Arbacia* eggs following various lengths of exposure to sea water 80 per cent. saturated with CO_2. Temperature, 21.2°–21.6°. Abscissa = time, in minutes, after insemination. Ordinate = percentage of eggs showing the first cleavage.

Symbol.	Length of Exposure.
● Dots	0 minutes
× Crosses	5 minutes
○ Circles	10 minutes
⊕ Crosses in circles	20 minutes
△ Hollow Triangles	35 (?) minutes
▲ Solid Triangles	80 minutes

For each of the six curves the final value, omitted from lack of space, was 100 per cent., except in the case of the twenty minutes exposure, where it was 98 per cent. There have also been omitted the cleavage curves following exposures of 160, 320, and 640 minutes, which ultimately showed cleavage in 97 per cent., 99 per cent., and 88 per cent. of the eggs respectively.

Table III. gives the delay in 50 per cent. cleavage corresponding to each cleavage curve of the complete series.

The relation between time of exposure and retardation of development is shown for a typical experiment in Table III., the values for 50 per cent. cleavage having been taken from the curves in Fig. 2.

Table III.

The Delay in 50 per cent. Cleavage Caused by Various Exposures to 80 per cent. CO_2.

(Experiment illustrated in Fig. 2.)

Exposure Time (in Minutes).	Delay in 50 per cent. Cleavage (in Minutes).
0	—
5′	5′
10′	12′
20′	18′
35′ (?)	34′
80′	86′
160′	186′
320′	370′
640′	750′

It will be observed that with the shorter exposures the recovery of the cleavage process may occur with great rapidity, the delay being but little more than the time corresponding to the period of exposure. Consequently, it has very often been found possible, in performing experiments, to predict the beginning of cleavage with a fair degree of accuracy simply by adding the exposure time to that required for the beginning of cleavage in the normal. As exposures become longer, the greater discrepancy between the exposure time and the delay of cleavage is wholly in one direction —that of prolonging the cleavage time. A further analysis of the data shows even in this respect a simple relation which may best be brought out by plotting the time required for 50 per cent. cleavage against the time of exposure, as has been done in Fig. 3. It will be noticed that straight lines may be drawn through the points representing any given experiment. Those indicated in the figure have been calculated by the method of least squares, with the result that the slopes of all the lines are approximately equal. All may be represented fairly accurately by the equation

$$y = a + 1.18x,$$

where a = the cleavage time of the eggs in the absence of CO_2; x = the time of exposure, and y = the cleavage time of the exposed eggs. Further data are given in Table IV.

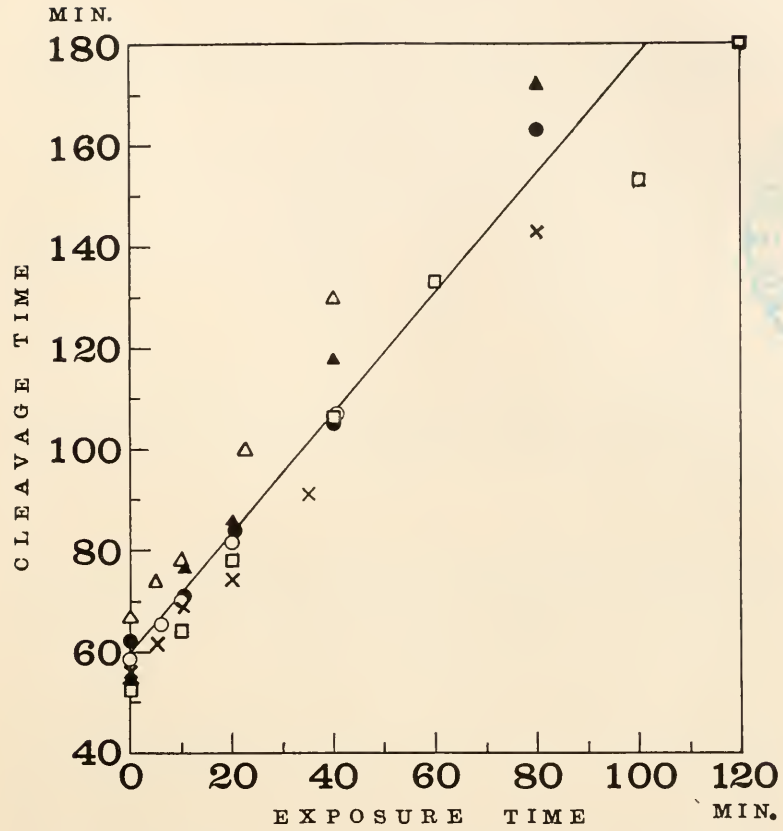

FIG. 3. The relation between the time required for 50 per cent. cleavage of *Arbacia* eggs and the duration of exposure to CO_2 at values from 30 per cent. saturation to practically complete saturation. Abscissa = minutes of exposure to CO_2. Ordinate = minutes required for cleavage in 50 per cent. of the eggs. Details are given in Table IV.

Symbol.	Experiment.
○ Circles	No. 119
× Crosses	No. 120
△ Hollow Triangles	No. 121
▲ Solid Triangles	No. 122
● Dots	No. 128
□ Squares	No. 129

(From lack of space, several values for exposures of over 120 minutes have not been included. These values were, however, used in calculating the equations the average of which—$y = 60.2 + 1.18x$—gives the straight line shown in this figure.) The slope of the curve would be unity, if recovery of the eggs were instantaneous upon their removal from CO_2.

Table IV.

Equations for the Retardation of 50 per cent. Cleavage in *Arbacia* Eggs in Sea Water 30 per cent. to "100 — " per cent. Saturated with CO_2.

Experiment.	CO_2 Content.	Oxygen Content.	Temperature.	Intercept.	Slope.
No. 119.....	"100 —"%	Trace	20.6°–21.2°	58.3	1.192
No. 120.....	80%	20%	21.2°–21.6°	52.6	1.182
No. 121.....	"100 —"%	Trace	20.4°–20.7°	71.	1.191
No. 122.....	"100 —"%	Trace	20.7°–20.8°	65.3	1.194
No. 128.....	40%	20%	20.8°–21.3°	59.35	1.23
No. 129.....	30%	20%	21.3°–22.4°	54.66	1.07
Mean Values.				60.2	1.18

(A preliminary experiment which showed a slope of 1.7 has been omitted from the average since this value differs widely from those subsequently obtained.)

The numerical value—1.18—of the slope of the lines in Fig. 3 shows that the retardation in cleavage produced by a given exposure is nearly, though not exactly, equal to the time of exposure. A value of unity would indicate exact equality. The fact that the slopes of the various lines are nearly the same is an indication of the general similarity, already mentioned, of the CO_2 effects at all tensions above 30 per cent. of saturation. The different values of the intercepts are without significance in this connection, since they represent merely the normal time of cleavage in those eggs which were not exposed to carbon dioxide.

Summary.

1. The first cleavage of the fertilized eggs of *Arbacia* is entirely suppressed, or practically so, in the presence of amounts of carbon dioxide greater than those corresponding to a 40 per cent. saturation of sea water or a tension of approximately 300 mm. Hg. In the presence of smaller amounts of carbon dioxide cleavage is possible, but is greatly delayed.

2. Since a very considerable oxygen deficiency causes only a slight delay in the cleavage process, the factor of oxygen lack is probably a negligible one in the results here described.

3. The effects of a complete suppression of the cleavage process

in sea water practically saturated with carbon dioxide are readily reversible up to exposures of twenty minutes. Beyond that point abnormalities may appear, though after exposures of two and one half hours 95 to 100 per cent. of the eggs ultimately divide.

4. The after effects of exposures of moderate length to carbon dioxide are comparatively slight, the delay in the first cleavage being only a little greater than the actual time of exposure. Mathematically, the relation

$$y = a + 1.18x$$

(where a = normal cleavage time; x = time of exposure; and y = cleavage time of the exposed eggs) has been found to describe fairly accurately the results obtained at 21.4° (± 1.°) with sea water from 30 per cent. saturation to almost complete saturation.

I am glad to have this opportunity to express my gratitude to Dr. M. H. Jacobs for his suggestion of this problem and for his continued interest in the progress of the work.

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ORIGIN AND DESCRIPTION OF BRISTLE IN *DROSOPHILA MELANOGASTER.*

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Origin and Description of Bristle in *D. melanogaster.*

In an experiment designed to test the combined effect of temperature and the genes linked with Curly upon crossing-over in the third linkage group, Dichæte females were mated with sepia spineless sooty rough Curly males. Among the 163 offspring of such a mating there appeared on April 11, 1925, two Dichæte females with the bristles on the head and thorax shorter (1/2 to 3/4 the length of corresponding bristles on the wild type) and slightly thicker than those in the wild type and slightly irregular in outline with a tendency to be twisted and truncate. The new character is called Bristle in the following account (Symbol Bl.). The fact that only two unusual females were found among so many made it seem improbable that the character difference depended upon a recessive or a sex-linked factor difference. Thus by exclusion, it was supposed that the mutant gene was dominant. Further the two individuals probably owed their appearance to a single mutation which may have occurred either in the late oogonial divisions of the female, or, in the spermatocyte or late spermatogonial divisions of the male parent. Since the male carried the dominant Curly and the female the dominant Dichæte it is more probable that the mutation occurred in the female since both individuals were not Curly.

These two females were mated with wild type males and their offspring (which were not counted) included Dichæte, Bristle, Dichæte Bristle and wild type flies. A number of the female Dichæte Bristle offspring were mated separately with homozygous Lobe males. Half the offspring were Bristle which supported the assumption that Bristle was dominant; half the Bristle individuals

were also Dichæte indicating that D and Bl are probably in different linkage groups (Table I.).

F_1 Dichæte Bristle Lobe males were mated with wild type females and F_1 Dichæte Bristle Lobe females were mated with wild type males. The male black-crosses showed no recombination of Bl and L so that Bl is in the second linkage group. The female back-crosses gave a cross-over value of 18.1 per cent. between L and Bl (Table I.).

TABLE I.

P_1 DICHÆTE BRISTLE ♀ × LOBE ♂.

L.	D L.	Bl L.	D Bl L.
158	146	142	134

B. C., F_1 DICHÆTE BRISTLE LOBE ♂ × WILD TYPE ♀.

L.	D L.	Bl.	D Bl.
218	188	200	169

B. C., F_1 DICHÆTE BRISTLE LOBE ♀ × WILD TYPE ♂ (DICHÆTE NOT CLASSIFIED).

L.	Bl.	Bl L.	Wild Type.
263	222	47	60

In order to find the approximate location of Bl in the second linkage group F_1 Bristle females from matings of Bristle × black purple curved were back-crossed to black purple curved males. The results (Table II.) show that Bl lies to the right of purple and give the following cross-over values b–p 6.5 per cent; p–Bl 0.18 per cent.; Bl–c 14.6 per cent. and p–c 14.8 per cent. (The standard values are b–p 6 per cent. and p–c 21 per cent. No explanation was found for the discrepancy in the purple-curved region.) As there were only three cross-overs between Bl and p, one of which was also a cross-over between b and p (a rare occurrence in 6.7 units) it seemed advisable to make further tests.

TABLE II.

P_1 BRISTLE ♀ × BLACK PURPLE CURVED ♂.

Bl. Wild Type.

191 225

B. C., F_1 BRISTLE ♀ × BLACK PURPLE CURVED ♂.

bpc-Bl.	bBl-pc.	bp-Blc.	bBlc-p.	bpBl-c.	bc-Blp.
725 576	33 55	97 122	10 5	1 1	0 1
1301	88	219	15	2	1

A black purple Bristle curved stock was made up by crossing the black purple Bristle female (which was also heterozygous for curved) from Table II. with a black purple curved male. F_1 Bristle females from mating of black purple Bristle curved × wild type raised at 31 degrees C. were back-crossed to black purple curved males for six days only with the results shown in Table III. The recombination percentages are as follows: b–p 14.9 per cent.; p–Bl 1.6 per cent.; Bl–c 23.7 per cent. and p–c 25.3 per cent. which correspond with the values b–p 14.0 per cent. and p–c 26.7 per cent. found by Plough ('17) for the same temperature.

TABLE III.

P_1. BLACK PURPLE BRISTLE CURVED ♀ × WILD TYPE ♂ AT 31° C.
F_1 Bristle ♀ × black purple curved ♂ 6 days only.

bpBlc- Wild Type.	bpBl-c.	bpBl-c.	bc-pBl.	bp-Blc.
167 198	35 25	40 70	12 13	4 5
365	60	110	25	9

That Bristle is lethal when homozygous was indicated when Bristle males and females (from Table I.) were inbred. Such matings gave approximately 2/3 Bristle and 1/3 wild type offspring (Table IV.). Further demonstration was obtained as follows: A black Bristle curved male (from Table II.) was mated to a wild type female and F_1 males and females were inbred. The eight curved and one black offspring out of 145 represent

cross-overs and show that homozygous Bristle is not viable. This is further shown by the inbreeding of F_1 Bristle males and females from a cross of Bristle purple (from Table II.) with wild type. No purple offspring appear because purple is so closely linked to Bristle. Bristle does not represent a deficiency toward the purple region at least. A balanced lethal stock with Bristle and Lobe linked, balanced against Curly has been made up and should prove of some value in quickly locating genes in the second linkage group.

Table IV.

Bristle ♀ × Bristle ♂ (from Table I.).

Bl.	Wild Type.
416	213

P_1, Black Bristle Curved ♂ × Wild Type ♀.
F_1 Bristle ♂ and ♀ inbred.

Bl.	Wild Type.	Blc.	bBl.
74	62	8	1

P_1 Bristle Purple ♀ × Wild Type ♂.
F_1 Bristle ♂ and ♀ inbred.

Bl.	Wild Type.
74	56

Summary.

1. A new bristle form in *Drosophila melanogaster* has been found and named Bristle, Bl.

2. The mutant gene is a dominant, lethal when homozygous.

3. The locus of Bl lies 0.18 per cent. to the right of purple at approximately 54.8 in the second linkage group.

4. A stock of Bristle Lobe balanced against Curly has been made up and is available for use.

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EXPERIMENTAL LOCALIZATION OF NEW AXES IN CORYMORPHA WITHOUT OBLITERATION OF THE ORIGINAL POLARITY.

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The various lines of evidence demonstrating the existence of physiological gradients in *Corymorpha* have been considered in earlier papers (Child and Hyman, '26, Child, '26*a*, '26*b*). In the last of these papers it was shown that the differential resulting from contact of one end of a stem piece with the bottom and free exposure of the other may determine the one as basal, the other as apical, irrespective of the original polarity. In accordance with this fact it was shown that in pieces undergoing reconstitution on the bottom the bipolar frequency is lower and the unipolar frequency higher than in those supported on loose cotton near the surface of the water so that the ends are more nearly equally exposed. In other papers it was shown that pieces after subjection to various inhibiting agents may develop new polarities and symmetries quite independent of the original axes and of the cut ends (Child, '27*a*, *b*). Apparently the inhibiting agents decrease or obliterate the original polarity and symmetry and the localizing influence of the cut ends and under these conditions the differential of position becomes more effective in localizing apical ends on the free surface and basal ends on the surface in contact or near the bottom.

The present paper is concerned with some further experiments on the determination of new polarities. In these experiments the new axes are localized as centers of high metabolic activity and growth without obliterating the original polarity.

Experimental.

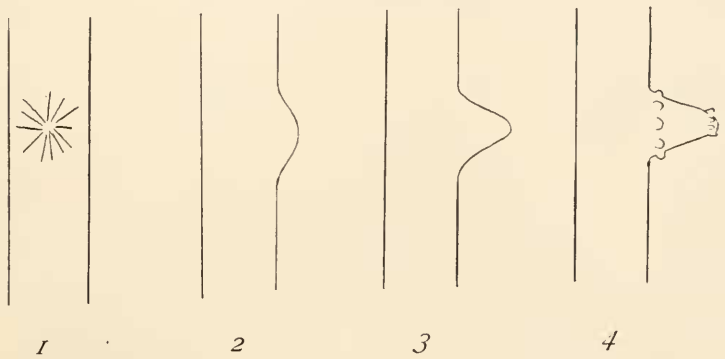
No indication of development of new hydranths by budding has been observed in *Corymorpha*. Among thousands of individuals

collected, only a single case of more than one hydranth on one stem has been observed. In this case the stem was single over most of its length, but divided into two distally and each of these bore a hydranth. The two hydranths were equal in size and it was not possible to distinguish one as terminal, the other as lateral. Another individual collected possessed a lateral stem outgrowth some ten mm. in length but without hydranth or base at its tip. A third individual possessed two manubria. These are the only cases of axial multiplication found thus far in the collected material. Considering the high frequency of such multiplication under experimental conditions (Child, '27 *a, b*) it seems remarkable that it does not occur more frequently in nature.

A simple lateral cut with smooth edges, extending a third or even half way through the stem closes within an hour or two under normal conditions and no new apical end or other outgrowth results from it. An earlier experiment on pieces of the actinian, *Harenactis,* suggested that a modification of the procedure employed in that case might determine a new polarity and symmetry in lateral stem regions of *Corymorpha.* In the case of *Harenactis* it was found that when mesenteries and muscles were much injured or in large part removed the shorter transverse pieces contracted in such a way as to bring distal and proximal cut edges of the body wall together and union took place between these edges about the whole circumference, giving rise to ' rings ' (Child, '09*b*). It was found further that in places where the union between the cut edges was smooth and without much new tissue no outgrowths developed along the line of union, while in places where more new tissue developed groups of tentacles appeared. This result led to the further experiment of mutilating opposite regions of the two cut edges by means of numerous small cuts close together and vertical to the edge. When these two mutilated regions came together they could not unite smoothly and extensive growth of new tissue took place before healing was complete. From this new tissue there gradually developed in some cases a new normal individual (Child, '10, Figs. 5, 6). The new apical region appeared only after complete closure of the wound by new tissue. This new tissue gradually bulged outward because of the internal water pressure, continued to grow and finally developed

as a new polar axis. The radial symmetry accompanying this new polarity seemed to be primarily merely an expression of the likeness of all radii in a plane vertical to the polar axis.

In the hope that it might be possible to determine a new polarity from the lateral stem region of *Corymorpha* in a manner somewhat similar to that employed in *Harenactis* the stems were cut as follows: with small, fine-pointed scissors cuts one to two mm. in length, radiating from a center, were made as indicated in Fig. 1, the purpose of the operation being merely to localize a region

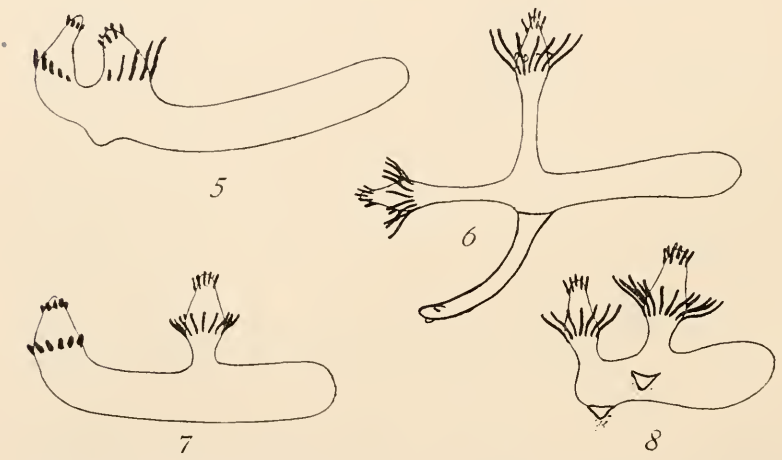


FIGS. 1–4. Development of hydranth from lateral region of growth determined by radiating cuts as shown in Fig. 1.

of active cells. In all cases the original hydranth was removed in order that its dominance might not interfere with the development of a lateral hydranth and in some of the earlier series the new hydranths which developed at one or both ends of the piece were also removed in early stages for the same reason, but this was found to be unnecessary. Closure of this wound was slower than in case of a simple cut, but was usually complete in twelve to twenty-four hours. In the successful operations the region began to bulge soon after closure (Fig. 2) and soon became a definite rounded outgrowth which underwent elongation (Fig. 3) and after two or three days attained the form of a hydranth with early stages of tentacles (Fig. 4).

Figures 5–18 show characteristic results of this operation. In all figures the region of stem covered with perisarc is indicated by heavier outline than other regions and the perisarcal accumula-

tion at the basal end is indicated by dotting. Fig. 5 is a case of new lateral polarity in a piece some twenty-five mm. in length from the middle of the naked region of a 70–80 mm. animal at a stage four days after operation. The apical hydranth of the piece is the second one developed, the first having been removed two days after section. The apical and the lateral hydranth are so near together that they mutually inhibit tentacle development on the sides facing each other and so have acquired a dorsoventrality with respect to each other. The side of the stem opposite the lateral hydranth shows an outgrowth which later becomes a base.



FIGS. 5–8. Development of new axes from lateral regions of growth determined by injury. Pieces 25 mm. in length from middle of naked region of animals 70–80 mm. Figures are about twice natural size. Figs. 5 and 6, two stages of a piece developing a complete new axis at right angles to the original polarity. Figs. 7 and 8, two stages of a piece which develops a new basal end in relation to both lateral and apical hydranth.

Fig. 6 represents the condition of the piece three days later. The two hydranths have now developed separate stems and a new base has arisen opposite the lateral hydranth. This development of a new individual from the apical end basipetally following the localization of the new apical end by the injury is an excellent example of apicobasal dominance. The localization of an active region by the injury determined a new hydranth, this determined successive stem regions basipetally until finally a new basal end

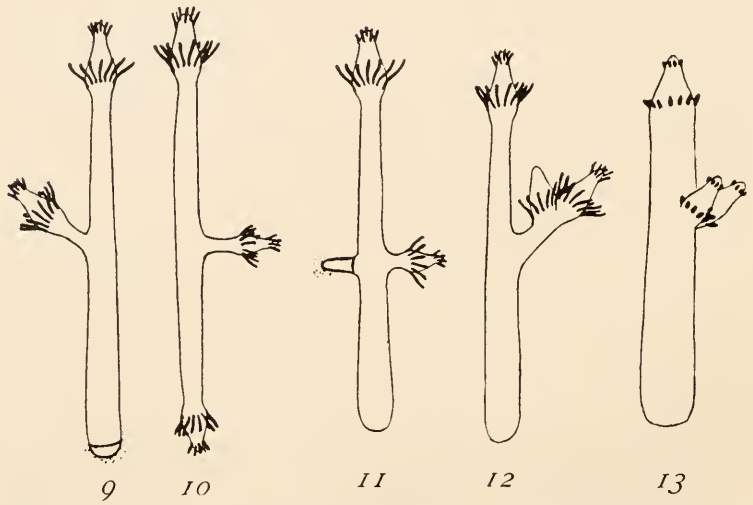
was determined on the opposite side of the piece, that is, the dominance of the new axis became effective through the stem at right angles to the original polarity. Contact with the substratum may have assisted in determining the lateral stem region as a basal end (Child, '27*a*, *b*, *c*), though this region was not found in contact when the piece was observed, but if the new polarity were not concerned in localizing the base it would probably have arisen at or near the proximal end of the piece. At this stage then the piece represents two distinct polarities at right angles to each other. In later stages the form became bipolar-unipolar, the new base becoming the base for both hydranths and the stem region proximal to the lateral polarity gradually undergoing resorption.

Fig. 7 shows another case from the same series at a stage four days after operation. Here also the first apical hydranth was removed and the figure shows the second developing. Fig. 8 shows the same form three days later. Lateral stem regions are developing into a new basal end in relation to each hydranth. In this case as in the preceding, contact may perhaps have been concerned in producing conditions favorable to base development from the side of the piece, but the localization of the two bases in relation to the hydranths indicates that the more distal regions of each axis were to some extent concerned in localizing the bases. In this case also the region of the stem proximal to the lateral hydranth was gradually resorbed and the form became biapical and bibasal. The two individuals would probably have separated completely like most other double forms if they had been kept long enough.

In both of these cases the proximal stem region apparently cannot maintain itself in the presence of the new lateral polarity and is resorbed. The new polarity obliterates the old, probably because the new represents higher levels of metabolism and so is able to grow at the expense of the older stem regions. Such growth of new axes at the expense of old stem regions has been observed frequently in other experiments (Child, '27*a*).

Figs. 9–13 show cases from another series in which the pieces included the whole or almost the whole length of the naked region of 50–60 mm. animals. The figures show stages five days after section. In Figs. 9 and 10 the lateral axes have not developed

basal ends, but in Fig. 11 a basal end is developing opposite the lateral hydranth and in Fig. 12 two hydranths have developed from the lateral outgrowth. Fig. 13, a piece of another series from 70–80 mm. animals three days after section is another case of two lateral hydranths.

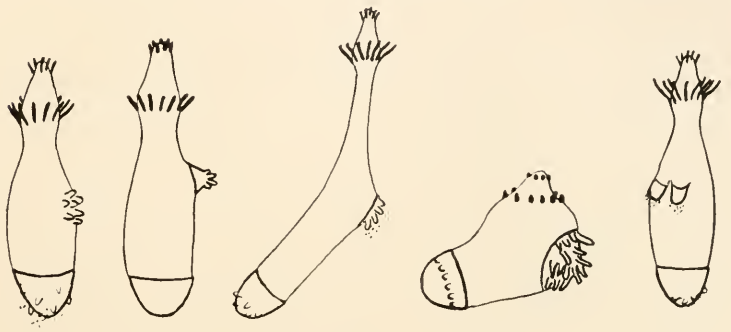


FIGS 9–13. Other cases of lateral hydranth development from region of growth determined by injury. Figs. 9–12 from pieces including the whole naked region of 50–60 mm. animals. Fig. 13 from piece including whole naked region of 70–80 mm. animal. The figures are slightly above natural size.

This lateral operation has been performed on thirty-five stem pieces and of these fifteen, or forty-three per cent. have given rise under standard conditions to new lateral axes consisting of at least a hydranth and more or less stem. Twenty pieces, fifty-seven per cent., healed without giving rise to new axes. Among the new lateral axes three, *i.e.*, nine per cent. of the total, became complete by the development of a basal end from the lateral stem region opposite the lateral hydranth.

It has been shown in earlier papers (Child, '26*b*, '27*a*, *b*), that either cut end or both, or any other region of a piece may develop as a basal end under inhibiting external conditions. In the light of these results it is to be expected that regions of lateral injury may also be made to develop as basal structures under inhibiting

conditions. Up to the present, however, only one experiment of this sort under inhibiting conditions has been carried out, but its results are conclusive. Of the twenty pieces each including the naked and half the perisarcal region, which were used in this experiment fourteen, seventy per cent. gave rise to basal structures in the region of lateral injury and one, five per cent., to a hydranth,



FIGS. 14–18. Cases of development under inhibiting conditions of basal structures from lateral growing region determined by injury. Pieces include whole naked and half perisarcal region of 50 mm. animals. Figures are about twice natural size.

while five, twenty-five per cent., healed without outgrowth. Figs. 14–18 show characteristic cases from this series four days after operation. In Fig. 14 the region of injury has developed merely a few holdfast or stolon buds, while in Figs. 15 and 16 stolon buds are present at the tip of a general basal outgrowth. In the case of Fig. 17 the stolon buds in the region of injury are more numerous and attain greater length than in any other case. In Fig. 18 the region of injury has given rise to two basal outgrowths. These cases are sufficient to demonstrate that the lateral injury may develop as a basal as well as an apical end.

DISCUSSION.

The experiments described above show that it is possible to localize new polarities in *Corymorpha* stems even without previously obliterating or decreasing the original polarity by means of inhibiting agents. It is important to note that these new polari-

ties resulting from lateral injury are not similar to the lateral partial discs which result from a transverse cut part way through the body in *Cerianthus* (Loeb, '91, Child, '05, '08) and *Harenactis* (Child, '09*a*). In those forms the opening remains because the cut edges of body wall and oesophagus unite and the new partial disc develops entirely on the proximal side of the cut, just as a disc develops on any distal cut end of a piece. In the case of *Corymorpha* the wound closes completely in the course of a few hours and it is only through the continued growth of the region after closure of the wound that the new axis is determined. If the injury does not initiate such growth no new axis develops.

These new lateral polarities are essentially induced buds and like other buds they give us important evidence concerning the origin and nature of new axes. If we observe, without theoretical prejudice, what happens in such a process, we see that the new axis originates as a local region of growth and becomes visible as an outgrowth of the body wall (Fig. 2) because the growth activity is evidently greatest in its middle region and decreases peripherally in all directions. The early rounded outgrowth undergoes elongation and the more active middle region necessarily becomes its tip (Fig. 3), in other words, the region of growth has now become a physiological axis characterized by a gradient in activity decreasing from the tip basipetally. There can be no doubt that when such a gradient is once determined in a particular kind of protoplasm the constitution of the protoplasm will play the chief part in determining its steepness, its length and the changes which it undergoes during development. If we admit this, it follows that however the gradient is determined its definitive form will be the same in a protoplasm of a certain constitution, consequently a gradient such as the one under consideration, determined by a local injury will determine the same course of development, *i.e.*, the same kind of an axis as the gradient in embryonic development, if the condition of the cells in the region of active growth is similar to that of embryonic cells of *Cormorpha*. If we take the facts as they stand it seems that there is no adequate reason to regard a polar axis in its simplest form as anything more than such a gradient as this. Experiment shows that when such a gradient is determined a new axis is determined and when the gradient is

obliterated the polarity is obliterated, so far as can be determined.

It has been shown for many forms, both plant and animal, that buds originate as gradients of this sort, resulting merely from the localization of an active region which is not sharply marked off from its surroundings but shows a gradient of decreasing activity from a central region toward the periphery. In consequence of differential growth, which itself results from the existence of this gradient, the radial gradient becomes an apicobasal gradient and a polar axis. There is no evidence to indicate that a polar axis is primarily anything more than such a dynamic differential with its structural protoplasmic correlates, or that differentiation along an axis requires anything more for its initiation than the quantitative differences at different levels of such a gradient.

The development of basal instead of apical structures from a lateral injury under inhibiting conditions is in complete agreement with the results of other experiments. It has been shown that the basal region of *Corymorpha* represents a secondary gradient which originates at the low end of the primary gradient (Child and Hyman, '26; Child, '26*a*). The high end of this secondary gradient, so long as it persists, is the basal tip and the slender modified stolons which constitute the holdfasts develop as lateral buds along this secondary gradient. These stolons show extremely rapid growth, but they originate only in regions of relatively low activity. When the activity of the region of lateral injury is decreased to a certain degree by inhibiting factors, the conditions must become more or less similar to those existing at the lower end of the primary gradient, and the lateral injury, like the lower end of the primary gradient, develops as a basal region. Whether a single basal outgrowth bearing stolon buds, or merely the stolon buds appear probably depends on various factors, *e.g.*, the degree of inhibition, the presence or absence of a definite growth region, etc. If a single general basal outgrowth arises the further development of the basal gradient and basal region follows in the same way as the development of the hydranth-stem gradient and region. Even if the central growth area resulting from the injury is not sufficiently well defined to determine a single general basal outgrowth, new stolon buds may be determined in relation to the entodermal canals or parts of canals in the injured region. Since the canals

have been mutilated by the injury the arrangement of the stolon buds is likely to be irregular, as in Figs. 14–18.

Development of two apical or basal ends from the region of injury is undoubtedly a result of determination by the injury of two regions of activity instead of one. Duplication of this kind has been very widely observed in many forms as a result of splitting or otherwise dividing a growing region into two.

One of the most interesting results of these experiments is the determination by the more distal levels of the new axis of a basal region on the opposite side of the stem where there is no injury (Figs. 5, 6, 8, 12). It is evident that the development of the distal region of the new axis has in some way altered conditions in the region of the stem which gives rise to the base, but it has been shown that contact or nearness to the bottom and the action of various inhibiting agents may alter conditions in the same direction in regions of the stem (Child, '26*b*, '27*a*, *b*). This being the case there is no good reason for supposing that the changes which initiate the development of the basal end are anything more than quantitative changes in physiological condition determined by the presence of the new gradient. In the case of Fig. 8 in which the apical hydranth takes up a more or less lateral position because of the position of the piece, it, as well as the lateral hydranth, develops a new basal end, perhaps with the assistance of the conditions resulting from contact of the region concerned with the bottom.

The new lateral axis develops the characteristic radial symmetry, except in cases such as Fig. 5, in which the differential resulting from proximity of the other hydranth determines mutual and opposed dorsoventrality. If we examine the facts, again without theoretical prejudice, it appears that the radial symmetry of the axis is primarily nothing more than likeness of all radii in a plane perpendicular to the polar axis. The primary growing region determined by the injury is more or less radially symmetrical because its activity decreases radially from a center and as it becomes a definite outgrowth (Figs. 2, 3) its radial symmetry appears to result from this radial differential and from the fact that a surface-interior differential exists at all points. With the localization of tentacles certain radii become different from others. The factors concerned in tentacle localization have been but little investi-

gated, but it is difficult to believe that the localization begins independently of external factors of some sort which determine where the first tentacle or tentacles shall appear. In the ordinary course of reconstitution the entodermal canals are important factors in localizing the new tentacles, as Torrey has shown (Torrey, '10). Factors concerned in tentacle localization in the lateral polarities have not been studied, but they will probably be found in the relations of the growing region to the rest of the stem. Study of *Hydra* and various hydroids indicates that localization of a single tentacle is sufficient to initiate the orderly development of others. Torrey's study of the order of appearance of tentacles in the embryonic development of *Corymorpha* is interesting in this connection as indicating that different localizing factors are concerned in different individuals for he finds that the process does not follow a uniform course (Torrey, '07). Apparently each region of growth, whether tentacle or other organ, dominates a certain area so that a similar organ cannot develop within that area. When a particular tentacle is localized, for example, another can develop only outside its range of dominance. Any part of the circumference in the tentacle forming region is undoubtedly capable of giving rise to a tentacle, but the actual localization in a particular case must depend on the factors concerned. That the outgrowth which becomes a polar axis with a radial symmetry can localize its own tentacles independently of any of the external differentials to which it is exposed is at least highly improbable and seems to require the action of some non-mechanistic ordering factor. If these observations and suggestions are correct, radial symmetry in these new axes has its origin in the primary likeness of radii at any particular level and in the difference between surface and interior which is present in some form in all organisms. The later localization of a series of similar organs in radial arrangement seems to demand the action of some differential external to the parts concerned.

Summary.

1. Buds have never been seen to arise from lateral stem regions in *Corymorpha* and a simple transverse cut into the side of the stem closes rapidly without development of a bud or other outgrowth, unless it extends almost through the stem.

2. A region of injury produced on the side of the stem by short cuts radiating from a center closes less rapidly than the simple transverse cut and in many cases gives rise after closure to a rounded outgrowth which becomes conical and develops into a new hydranth. This hydranth develops a stem at the expense of the old stem and in some cases the new axis determines a new basal end on the opposite side of the piece, thus completing a polarity at right angles to the original axis. Occasionally two hydranths instead of one are localized by the injury.

3. The experimental data indicate that the new polarity and symmetry are the necessary consequence of the localization of a center of cellular activity. The radial gradient of decreasing activity from the center peripherally becomes, as growth proceeds, the axial gradient and the radial symmetry is primarily merely a similarity of all radii vertical to the polar axis at any level.

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OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

VOL. LIII JULY, 1927 NO. 1

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PUBLISHED MONTHLY BY THE
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PUBLISHED MONTHLY BY THE

MARINE BIOLOGICAL LABORATORY

PRINTED AND ISSUED BY

LANCASTER PRESS, INC.

LANCASTER, PA.

AGENT FOR GREAT BRITAIN

WHELDON & WESLEY, LIMITED

2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2

Single Numbers, $1.00. Per Volume (6 numbers), $4.50

Entered October 10 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16 1894

All communications and manuscripts should be sent to the Managing Editor, the University of Chicago, Sept. 15th to June 15th, or to Biological Bulletin, Woods Hole, Mass., June 15th to Sept. 15th. Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa.

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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

VOL. LIII DECEMBER, 1927 No. 6

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